UNIVERSITY OF LIVERPOOL

INVESTIGATION OF THE ROLE OF INFLAMMATION IN DRUG-INDUCED HEPATOTOXICITY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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DECLARATION

This thesis is the result of my own work. The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or qualification.

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CONTENTS

ABSTRACT

Drug-induced liver injury (DILI) is the most frequent cause for withdrawal of approved drugs and attrition of drug development. Although the cellular mechanisms of DILI leading to overt tissue and organ damage are still not fully defined, model hepatotoxins such as acetaminophen (paracetamol; APAP) have been used in animal models to provide important insight into the relationship between chemical structure, drug metabolism and cellular response. The overall extent of APAP injury is thought to be dependent on the activation of cell-cell signalling pathways, in particular between hepatocytes and cells of the innate immune system. However, the extent of the role of the innate immune and inflammatory response in the modulation of liver injury remains poorly defined, with conflicting evidence in the literature. The work presented in this thesis has used model hepatotoxins APAP and galactosamine (GalN) and the innate immune stimulator lipopolysaccharide (LPS) as tools to explore the mechanism of inflammation associated with DILI with the aim to further understand the various inflammatory pathways during DILI and their contribution to the progression of liver injury.

Male C57BL/6 mice were used to investigate the inflammatory response associated with APAP, GalN and LPS exposure. Inflammation was measured as serum levels of TNF-α, IL-6 and IL-1β by ELISA and neutrophil infiltration as assessed by histological analysis. Hepatotoxicity in the APAP model was characterised by a dose and time-dependent increase in serum ALT and was confirmed by histological analysis (0-1000mg/kg; 0-24hr). Significant elevations in levels of TNF-α, IL-6 and IL-1β were observed in the serum of mice following APAP overdose (530mg/kg; 0-24hr). Overnight fasting of mice led to a greater hepatotoxic and inflammatory response compared to non-fasted mice post APAP treatment. The LPS model was characterised by having no hepatotoxicity, as serum ALT activity remained at control levels (control – 41.37±8.45U/l; LPS – 50.57±5.56U/l; 5mg/kg; 24hr) and having a potent inflammatory response as determined by cytokine release (TNF-α – 513.13±50.15pg/ml; IL-6 – 1201.12±157.20pg/ml; IL-1β – 227.08±39.93pg/ml; 5mg/kg; 24hr) and neutrophil infiltration. The GalN model was characterised by mild hepatotoxicity (ALT activity 875.30±144.81U/l; 800mg/kg; 24hr) and a mild inflammatory response, with increase in cytokines (TNF-α – 56.44±5.68pg/ml; IL-6 – 63.22±8.41pg/ml; IL-1β – 40.72±3.64pg/ml; 800mg/kg; 24hr) and neutrophil infiltration. Concurrent administration of suppressors of either metabolism (DMSO), or the innate immune response (aspirin, ethyl pyruvate and anti-HMGB1 antibody) was also investigated. DMSO inhibited APAP metabolism, whereas aspirin had no effect on APAP toxicity or the inflammatory response. Delayed administration of ethyl pyruvate reduced toxicity and serum HMGB1 content post APAP. Expression of RAGE mRNA over a 24hr time course was measured in whole livers of fasted and fed mice treated with APAP by RT-PCR. Full-length (flRAGE), endogenous secretory (esRAGE) and total (tRAGE) isoforms of RAGE increased in fed mice between 1hr and 3hr. Fasted mice showed a more prolonged increase in expression of all 3 isoforms of RAGE, culminating in a peak increase at 5hr. Western blot analysis determined that there were no changes in RAGE protein expression in whole liver over a 24hr period of APAP

exposure. In situ hybridisation revealed a nuclear accumulation of RAGE mRNA in hepatocytes following APAP exposure.

In summary, the studies described in this thesis have provided further evidence for the role of inflammation during DILI by using and developing a variety of *in vivo* models. These models assessed both basic and novel mechanisms of inflammation, such as cytokine release, hepatic neutrophil infiltration and RAGE expression, and tested potential therapeutic inhibitors such as aspirin and EP.

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PUBLICATIONS

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ABBREVIATIONS

α	Alpha
ADR	Adverse drug reaction
ALF	Acute liver failure
ALT	Alanine aminotransferase
AP	Alkaline phosphatase
APAP	Acetaminophen; paracetamol
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
°C	degrees centigrade
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
COX2	Cyclooxygenase 2
CYP450	Cytochrome P450
dH_2O	Distilled water
DAB	3,3'-diaminobenzidine
DAMP	Damage-associated molecular pattern
DEPC	Diethyl pyrocarbonate
DILI	Drug-induced liver injury
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene, Plasticizer and Xylene
DTT	Dithiothreitol
EDTA	Ethylenedinitrilotetracetic acid
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular regulated kinase
FBS	Fetal bovine serum
γ	Gamma
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCLC	γ-glutamylcysteinesynthetase catalytic subunit
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O	Water

H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulphonic acid
HMGB1	High mobility group box 1
HO-1	Heme oxygenase1
hr	Hour
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IκB	Inhibitor of kappa B
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneal administration
ISH	*In Situ* Hybridisation
i.v	Intravenous administration
JNK	c-Jun N-terminal kinase
κ	Kappa
KC	Kupffer Cell
kDa	Kilodalton
Keap1	Kelch-like ECH-associated protein-1
L	Litres
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
mg	Milligrams
µg	Micrograms
ml	Millilitres
µl	Micromolar
mM	Millimolar
µM	Micromolar
MOPS	2-(*N*-morpholino) propane sulphonic acid
MQ	Milli-Q water
mRNA	Messenger RNA
NAC	N-acetylcysteine
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine di nucleotide phosphate (reduced)

NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NAPQI	*N*-acetyl-*p*-quinonimine
NBT	Nitro blue tetrazolium
NF-κB	Nuclear factor-κB
NHAIR	No histological abnormality is recognised
NK	Natural killer cells
NKT	Natural killer cells with T cell receptors
NLR	Nod-like receptors
NLS	Nuclear localisation sequence
Nrf2	Nuclear factor-erythroid 2-related factor 2
NSAID	Non-steroidal anti-inflammatory drug
NSS	Neutral sheep serum
O_2	Molecular oxygen
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI	Protease inhibitor
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCC	Sodium chloride citrate
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sRAGE	Soluble RAGE
TBS	Tris buffered saline
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TNF-R	Tumour necrosis factor receptor
T-TBS	Tween-Tris buffered saline
U	Units
UV	Ultraviolet
V	Volts
v/v	Volume per volume
w/v	Weight per volume

CHAPTER ONE

GENERAL INTRODUCTION

CONTENTS

1.1 INTRODUCTION

Adverse reactions to drugs are one of the main causes of hospital admission (Pirmohamed et al., 2004), leading to patient morbidity and mortality and are largely responsible for drug attrition during development (Park et al., 2005). Whilst adverse drug reactions affect every organ system within the body, the liver is one of the most commonly affected organs. Drug-induced liver injury (DILI) has proven to be a major problem for both the pharmaceutical industry and regulatory authorities and is the most frequent cause for withdrawal of approved drugs and attrition of drug development. 10.2% of 548 new chemical entities approved between 1975 and 1999 in the United States acquired a new black box warning or were withdrawn (Lasser et al., 2002).

It has been reported that 50% of acute liver failure cases in the United States are as a result of drug-induced liver toxicity, with more than 800 drugs associated with hepatotoxicity (Dossing et al., 1993; Ostapowicz et al., 2002). The liver is the main site for metabolism of endogenous and exogenous compounds, making it a common target for drug-induced toxicities. One of the liver's main physiological roles is the clearance and metabolism of xenobiotics into hydrophilic metabolites in order to facilitate their excretion. However, drug metabolism can lead to the formation of chemically reactive species that have the potential to damage cellular macromolecules. Although the cellular mechanisms of DILI leading to overt tissue and organ damage are still not fully defined, it appears to involve 2 pathways; direct hepatotoxicity and adverse immune responses. Model hepatotoxins have been used in animal models to provide important insight into the relationship between chemical structure, drug metabolism and cellular response. One classically studied drug used to examine the mechanisms of hepatotoxicity is acetaminophen (paracetamol; APAP).

APAP is a popular analgesic that is safe at therapeutic doses but can produce centrilobular hepatic necrosis in cases of overdose, which may lead to acute liver failure. APAP hepatotoxicity has been well characterised in animal models, where it has been defined that toxicity is mediated through the formation of an electrophilic reactive metabolite N-acetyl-p-benzoquinonimine (NAPQI) (Mitchel et al., 1973a), which depletes intracellular stores of hepatic glutathione (GSH) and initiates covalent binding to cellular macromolecules. This

leads to cellular dysfunction and oxidative stress and may eventually lead to cellular damage and death. In most instances in DILI, it appears that hepatocyte damage and death can trigger the activation of other cells, which can initiate an inflammatory response. This event may overwhelm the liver's capacity to repair and regenerate, thereby contributing to the pathogenesis of liver injury.

Despite intense research, the extent of the role of the innate immune and inflammatory response in the modulation of liver injury remains poorly defined. It is reported that hepatocyte stress or damage can result in the release of signals that stimulate activation of other cells, particularly those of the innate immune system such as Kupffer cells (KC), natural killer cells and NKT cells. These cells can contribute to the progression of liver injury by producing proinflammatory mediators and chemokines to further recruit inflammatory cells such as neutrophils to the liver (Laskin et al., 1986; Luster et al., 2001; Liu et al., 2004; Holt et al., 2006; Ishida et al., 2006; Jaeschke et al., 2006). In contrast to this, innate immune cells also represent a major source of hepatoprotective factors such as IL-6 and IL-10 as it has been demonstrated that transgenic mice lacking these are more susceptible to APAP-induced liver injury. Thus, a balance of proinflammatory and hepatoprotective mediators produced after activation of the innate immune response could determine the susceptibility and extent of liver injury after toxic insult.

The contribution of the signals released by dying hepatocytes to the severity and progression of DILI is also of interest. The release of high mobility group box 1 protein (HMGB1) by necrotic cells, but not apoptotic cells, has been implicated as an important factor in connecting necrotic cell death with the innate inflammatory response (Scaffidi et al., 2002). HMGB1 is classed as a damage-associated molecular pattern molecule (DAMP) that acts as a 'danger' signal to cells of the innate immune system following cell damage (Hori et al., 1995; Yu et al., 2006; Tian et al., 2007). HMGB1 can trigger an inflammatory response by signalling through its receptors; toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE). These signalling pathways could provide a potential target for investigating the role of the innate immune and inflammatory response to the pathogenesis of DILI.

The overall aim of this thesis was to assess the contribution of the inflammatory response in the progression of DILI by targeting certain points in the signalling pathway between dying hepatocytes and cells of the innate immune system. The hepatotoxins APAP and galactosamine (GalN) and the innate immune stimulator lipopolysaccharide (LPS) have been investigated to further understand the mechanisms behind drug-induced inflammation and hepatotoxicity. Targeting the signalling pathway between dying hepatocytes and inflammatory cells will provide help in the understanding of how hepatotoxins trigger an inflammatory response which can ultimately exacerbate the initial liver injury.

1.2 ADVERSE DRUG REACTIONS

Adverse drug reactions (ADRs) represent a significant health problem and are a major cause of patient morbidity and mortality. In the United States it was estimated that in 1994, over 100,000 patients died from ADRs (Lazarou et al., 1998). A prospective analysis of hospital admissions from two large Merseyside general hospitals in the UK, conducted over a 6 month period during 2001-2002 showed that ADRs accounted for 1225 (6.5%) out of 18,820 admissions. 28 (2.3%) of those patients admitted due to ADRs, died as a direct consequence of the reaction (Pirmohamed et al., 2004). The projected annual cost of such admissions to the National Health Service (NHS) was £466m. ADRs are largely responsible for the high attrition rate of potential new drugs within the pharmaceutical industry. It was reported that 10.2% of 548 new chemical entities approved between 1975 and 1999 in the United States acquired a new black box warning or were withdrawn (Lasser et al., 2002).

ADRs can be classified in terms of their clinical, pharmacological and chemical characteristics (Park et al., 1998) (Table 1.1). Type A reactions are predictable from the known pharmacology of the drug and are usually dose-dependent. These can usually be avoided by dose reduction and are rarely life-threatening. An example of a type A reaction is bleeding with anticoagulants such as warfarin. In contrast, type B reactions cannot be predicted from the basic pharmacology of the drug, are often independent of dose and are usually uncommon and therefore referred to as idiosyncratic. Although less common than type A reactions, type B reactions have the potential to be serious and even life-threatening. Examples of type B reactions include halothane hepatitis and anticonvulsant hypersensitivity.

Type C reactions can be predicted by the chemical structure of the drug or metabolite. An example of this type of reaction is paracetamol hepatotoxicity. The different types of ADRs are diverse and are known to affect every organ system in the body. As a result of patient morbidity and mortality, drug-induced liver injury (DILI) has proven to be a major problem for both the pharmaceutical industry and regulatory authorities, as it is the most frequent cause for post marketing withdrawal of medications despite pre-clinical and clinical screenings (Jaeschke et al., 2002).

Table 1.1: Classification of ADRs (Park et al., 1998)

Reaction Type	Characteristics	Examples
A - Augmented	Predicted from drug pharmacology Dose-dependent Resolve by dose diminution	Hypotension with antihypertensives and haemorrhage with anticoagulants
B - Idiosyncratic	Not predictable from the drug pharmacology Not dose-dependent Patient-specific Metabolism or immune components dictate individual susceptibility	Halothane hepatitis and anticonvulsant hypersensitivity
C - Chemical	Can be predicted or rationalised from the chemical behaviour of the drug or its metabolite	Paracetamol hepatotoxicity

1.3 DRUG-INDUCED LIVER INJURY

While ADRs can affect every organ system in the body, the liver represents a primary target for adverse drug reactions due to its central role in biotransformation and excretion of foreign compounds. Drug-induced liver injury (DILI) is attributed to a significant cause of patient morbidity and mortality (Lee et al., 2005) and accounts for more than 50% of acute liver failure in the United States, 39% of which includes hepatotoxicity caused by APAP (Ostapowicz et al., 2002). DILI is a major cause of drug attrition during development and the most frequent reason for the withdrawal of approved drugs from the market (Lee, 2003). An

estimated 1000 drugs have been implicated in causing liver damage on more than one occasion (Kaplowitz., 2004).

DILI can affect all cells of the liver which can lead to a variety of pathological conditions, including acute and chronic hepatitis, fibrosis/cirrhosis, cholestasis and hepatic artery/vein damage (Larrey et al., 2000). The predominant forms of DILI are acute hepatitis and cholestasis, of which the former is characterised by a marked increase in aminotransferases coinciding with hepatocellular necrosis (Gunawan., 2004). Current methods for the detection of liver damage are through the measurement of serum or plasma aminotransferases, which are enzymes released from the liver during necrosis. They are universally important indicators for studies ranging from preclinical animal studies to post-marketing patient monitoring (Amacher., 1998). One of the most commonly measured enzymes is alanine aminotransferase (ALT), with severe liver damage represented by levels exceeding 1000U/l (Beckett et al., 1985). A major limitation to using this enzyme as a marker of liver toxicity is that it is not an early biomarker, as it is only present once overt liver toxicity and necrosis have occurred.

1.3.1 Mechanisms of DILI

The mechanism of DILI appears to involve 2 pathways, direct hepatotoxicity and immune mediated damage. The pathogenesis of DILI can be related to the chemical properties of the parent drug or its metabolite. DILI in some instances can be initiated by bioactivation of drugs to chemically reactive metabolites, which have the ability to interact with cellular macromolecules leading to cellular dysfunction. The impairment of hepatic cellular function can ultimately lead to cell death and possible liver failure. Hepatic cellular death and dysfunction have the ability to initiate immunological response, triggering both innate and adaptive immune response. Hepatocyte damage or stress can result in the release of signals that stimulate the activation of other cells, particularly those of the innate immune system such as Kupffer cells (KC), natural killer cells and NKT cells. These cells can contribute to the progression of liver injury by producing proinflammatory mediators and chemokines to further recruit inflammatory cells such as neutrophils to the liver (Blazka et al., 1995; Blazka et al., 1996; Ishida et al., 2002).

1.3.2 Physiological role of drug metabolism

Drug metabolism is the process by which drug molecules are chemically altered by enzymatic biotransformation, usually into more hydrophilic metabolites in order to facilitate their excretion. The liver is the principle organ and quantitatively the most important site of drug metabolism. Xenobiotics are usually non-polar, lipophilic chemical compounds that are readily absorbed in the gastrointestinal tract. Metabolism is important to increase the hydrophilicity and facilitate their excretion in the urine or bile. Drug metabolism can be divided into 3 phases; phase I (functionalisation/bioactivation), phase II (conjugation/detoxification) and phase III (transport/excretion) (Pachkoria et al., 2007). Phase I reactions result in the exposure or incorporation of a functional group that is then a target for phase II conjugation reactions to form a highly polar conjugate (Guengerich & Shimada., 1991). Phase I reactions do not necessarily occur before phase II reactions. A drug can be metabolised by phase II metabolism without having previously being involved in phase I metabolism, providing there is a suitable functional group present on the parent compound (Gibson, 1994). Phase III metabolism involves the transport of drug conjugates across membranes (Pachkoria et al., 2007). While metabolism results in detoxification of many xenobiotics, some drugs are bioactivated to reactive and potentially toxic metabolites (Grillo et al., 2003). An imbalance between the three phases could lead to an increase of reactive metabolites resulting in liver toxicity (Pachkoria et al., 2007).

1.3.3 Phase I metabolism and involvement in DILI

Phase I metabolic reactions are also known as functionalisation reactions and incorporate or unmask functional groups, such as hydroxyl groups (-OH), primary amines ($-NH_2$), sulphydryl (-SH) and carboxylic acids (-COOH) by oxidation, reduction, hydrolysis or hydration reactions to facilitate phase II reactions (Gibson, 2001). Phase I drug metabolism enzymes include the microsomal cytochrome P450 monoxygenase system (CYP450) enzymes which are found in high abundance in the liver and catalyse the majority of phase I oxidation reactions (Timbrell, 2000). The CYP450 family are membrane-associated enzymes located on the inner mitochondrial membrane or in the endoplasmic reticulum of cells (Meyer et al., 1996; Guengerich et al., 2003). They have been referred to as 'mixed function

oxidases' due to their wide substrate specificities. The primary role of CYP450 enzymes is to act as a detoxification pathway and to metabolise endogenous and exogenous compounds. However, there is the possibility that reactions catalysed by CYP450 enzymes may result in liver injury as they may generate metabolites that are more reactive and toxic than the parent compound. This can result in liver damage in several ways. Metabolism of drug molecules can generate free radicals, leading to an increase in cellular oxidative stress. The exposure or incorporation of functional groups can lead to generation of electrophiles that may be detrimental to biological systems by potentially reacting with electron rich macromolecules such as proteins, DNA and RNA by covalent interactions. The chemical modification of these cellular macromolecules has the potential to render it toxic such as tissue necrosis, cellular apoptosis, chemical carcinogenesis and hypersensitivity.

1.3.4 Phase II metabolism and involvement in DILI

Phase II metabolism involves the conjugation of a hydrophilic chemical moiety to a drug molecule, thereby increasing its polarity and water solubility to aid excretion (Timbrell, 2000). The addition or exposure of a functional group by phase I reactions enables the conjugation of an endogenous substrate to form a highly polar and non-reactive conjugate, that can be more readily excreted than the parent compound. Phase II reactions consist of a diverse group of enzymes such as UDP-glucuronosyltransferases (UGT), N-acetyltransferases (NAT), sulphotransferases (SULT) and glutathione S-transferases (GST) (McCarver & Hines., 2002). An important function of phase II enzymes is the detoxification of reactive molecules that may be generated by phase I drug metabolism (Park et al., 2005). This enables the excretion of drugs into the urine or bile. Phase II reactions include glucuronidation, sulphation, acetylation, methylation, amino acid conjugation and glutathione (GSH) conjugation (Zamek-Gliszczynski et al., 2006).

GSH conjugation represents one of the most protective processes in the liver in the detoxification of reactive species generated as a result of metabolism. GSH is an endogenous tripeptide (γ-glutamylcysteinylglycine) consisting of glutamate, cysteine and glycine amino acids and is the most abundant non-protein cellular thiol, present in millimolar concentrations. It is an important antioxidant, reductant and scavenger within the cell (Hayes

et al., 1999) and exists in two forms, oxidised (GSSG) and reduced (GSH). GSH can conjugate either directly with a reactive electrophilic species or by facilitation through GST forming a thioether bond between the cysteine residue of GSH and the electrophile (Coles et al., 1988). GSTs are a family of enzymes located in the cytosol and microsomes. Cytosolic GSTs (95%) are encoded by at least 5 gene families whereas the membrane bound microsomal GSTs are encoded by one gene (Meister and Anderson, 1983; Dirven et al., 1995). The conjugation process with GSH is of particular importance when sulphation and glucuronidation pathways become saturated (Timbrell, 2000; Gibson, 2001). In cases where GSH depletion occurs, such as that seen with highly reactive metabolite exposure, other thiol groups, especially those on critical proteins become vulnerable to attack with subsequent oxidation, cross-linking and formation of covalent adducts. Protein sulphydryl groups appear to be the most susceptible nucleophilic target for electrophilic attack. Often these sulphydryl groups are critical for enzyme function. GSH depletion has been shown to be a prerequisite for the onset of toxicity (Mitchel et al., 1973b). An example of this is during APAP hepatotoxicity, when synthesis of new GSH is not capable of replenishing GSH used through conjugation or oxidation to GSSG (Mitchel et al., 1973b). Although Phase II metabolism is principally a detoxification pathway there is still a potential for bioactivation, which can lead to DILI. UGT catalyses the conversion of carboxylic acid containing drugs, such as non-steroidal anti-inflammatory drugs (NSAID), to acyl glucuronides that have the potential to bind covalently to plasma and hepatic proteins (Zhou et al., 2005).

1.3.5 Phase III metabolism and involvement in DILI

Phase III involves hepatic drug removal, involving a number of drug transporter proteins (Ishikawa, 1992). Mizuno et al. (2003) cloned and characterised plasma membrane-bound hepatocyte transporters and revealed that many drugs enter and exit hepatocytes by energy-dependent transporters rather than by diffusion. These transporters play a role in the transportation and removal of endogenous and exogenous compounds (Borst et al., 2002). The predominant transporters involved in drug uptake belong to the solute carrier (SLC) transporter superfamily, located on the sinusoidal plasma membrane. Examples include organic anion transporters (OAT) (Reichel et al., 1999), organic cation transporters (OCT) (Zhang et al., 1998) and sodium taurocholate cotransporting polypeptide (NTCP) (Hagenbuch

et al., 1994). The transporters involved in the excretion of drugs and their metabolites from hepatocytes are members of the ATP-binding cassette (ABC) transporter superfamily located on the biliary canalicular plasma membrane, where they mediate the excretion of drugs into bile, or on the sinusoidal membrane, where they mediate efflux back into the circulation (Dean et al., 2001; Kerb et al., 2001). Examples of these include multi-drug resistance protein (MDR), breast cancer resistance protein (BCRP) and bile salt export pump (BSEP). Inhibitors of these export proteins or genetic polymorphisms which alter protein function have the potential to induce cholestatic injury. The liver injury produced by compounds such as sulindac, flucloxacillin and terbinafine is attributable to the inhibition of canalicular bile salt transporter (Bolder et al., 1999; Iverson and Uetrecht, 2001; Lakehal et al., 2001).

1.3.6 Drug-induced cell death

The mechanisms leading to drug-induced cell death are still not fully understood but what is known is that the occurrence of hepatic cell necrosis and apoptosis are events which follow the capacity of the liver to respond to toxic insults (Grattagliano et al., 2002).

Apoptosis is a mechanism of programmed cell death that is executed during normal physiological regulation of cell number (Kerr et al., 1972) and can occur when a cell is damaged beyond repair to prevent the spread of damaged DNA to live cells. Hallmarks of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (Robertson and Orrenius, 2000). The cells fragment into apoptotic bodies that are phagocytosed by adjacent cells and macrophages for lysosomal degradation, minimising the leakage of cellular components into the extracellular space and preventing the induction of subsequent inflammatory responses (Savill et al., 2003). Many stimuli can initiate apoptosis such as tumour necrosis factor alpha (TNF-α) or Fas ligand (FasL) that engage with death receptors on the cell surface (Ashkenazi et al., 1998; Latta et al., 2000). Usually these stimuli lead to activation of a cascade of cysteine-aspartate proteases (caspases), which cleave cellular proteins after an aspartate residue (Cohen, 1997) leading to the degradation of the cell.

Unlike apoptosis, necrosis is regarded as the accidental or uncontrollable form of cell death and is a much less orderly process. Necrosis occurs as a consequence of acute cellular injury and results in the release of intracellular contents after cellular membrane damage. With respect to DILI, the release of intracellular contents can trigger an inflammatory response, leading to exacerbation of injury (Robertson and Orrenius, 2000). Necrosis is characterised by mitochondrial swelling, loss of plasma membrane integrity and nuclear DNA being randomly cleaved as a consequence of cellular degradation. APAP is a model hepatotoxin that has been shown to result in necrotic hepatocyte cell death (Jaeschke et al., 2003a).

The processes of apoptosis and necrosis during liver injury are not mutually exclusive, as the typical response of tissues and cells to injurious stresses and other death signals is a mixture of events associated with apoptotic and necrotic cell death which can occur in parallel or sequentially.

1.4 ACETAMINOPHEN HEPATOTOXICITY

Acetaminophen (Paracetamol; APAP) is a widely used analgesic and antipyretic which is safe at therapeutic doses of 4g/day (Bray, 1993) but is associated with acute liver failure (ALF) caused by massive centrilobular necrosis after an overdose (Davidson et al., 1966; Vermeulen et al., 1992). The main mechanism of action is considered to be through the inhibition of cycloxygenase (COX) activity and a reduction in prostaglandin synthesis (Botting et al., 2000; Boutaud et al., 2002). APAP-induced hepatotoxicity continues to be a significant cause of patient morbidity and mortality and is the single biggest cause of acute liver failure in the UK and USA (Ostapowicz et al., 2002; Hawkins et al., 2007; Khashab et al., 2007). APAP hepatotoxicity has been widely studied as it provides a clinically relevant tool in which to study the toxicological consequences of drug metabolism in *in vitro* and *in vivo* models.

1.4.1 Role of drug metabolism in APAP hepatotoxicity

APAP is extensively metabolised in the liver (Mitchel et al., 1973a; Lee et al., 1996). At therapeutic doses, around 55% and 30% of APAP is bioinactivated to glucuronide and

sulphate conjugates, by UDP-glucuronosyltransferase (UGT) and sulphotransferase (ST), respectively (Howie et al., 1977). These metabolites can then be rapidly excreted in the urine (Nelson, 1990). A small proportion of the therapeutic dose of APAP (5-10%) is bioactivated to the reactive metabolite N-acetyl-p-benzoquinonimine (NAPQI) by several CYP450s, primarily CYP2E1 and also CYP3A4 and CYP1A2 (Dahlin et al., 1984; Raucy et al., 1989; Thummel et al., 1993). NAPQI is conjugated to GSH resulting in its detoxification and is excreted in the urine as cysteine and mercapturate conjugates (Mitchel et al., 1973a) (Figure 1.1).

During overdose, the glucuronidation and sulphation pathways become saturated, forcing a greater fraction of the dose to become metabolised via the oxidative pathway. This results in an increase in the formation and subsequent accumulation of NAPQI, exceeding the cellular stores of hepatic GSH and new GSH synthesis, leading to depletion of hepatic GSH (Mitchel et al., 1973a; Mitchel et al., 1973b; Potter et al., 1973; Dahlin et al., 1984; Davies et al., 1986; Lee at al., 1996). The accumulation of NAPQI has been shown to result in binding of NAPQI to cellular proteins (Guengerich et al., 1985). As a result of binding to cellular proteins, NAPQI is capable of damaging the cell through oxidative stress, mitochondrial dysfunction and DNA damage. The covalent modification of proteins and the oxidation of protein thiols are believed to correlate with APAP-induced toxicity (Davis et al., 1974; Potter et al., 1974; Roberts et al., 1987; Bartolone et al., 1988). The standard treatment for APAP intoxication is the administration of N-acetylcysteine (NAC), which replaces hepatic GSH after depletion and prevents toxicity. NAC is most beneficial if given within 16hrs after APAP overdose. Without NAC the depletion of GSH stores and covalent binding of proteins by NAPQI will not only result in cellular toxicity (David Josephy, 2005) but also activate the cellular adaptive defence response during drug exposure (Park et al., 2005).

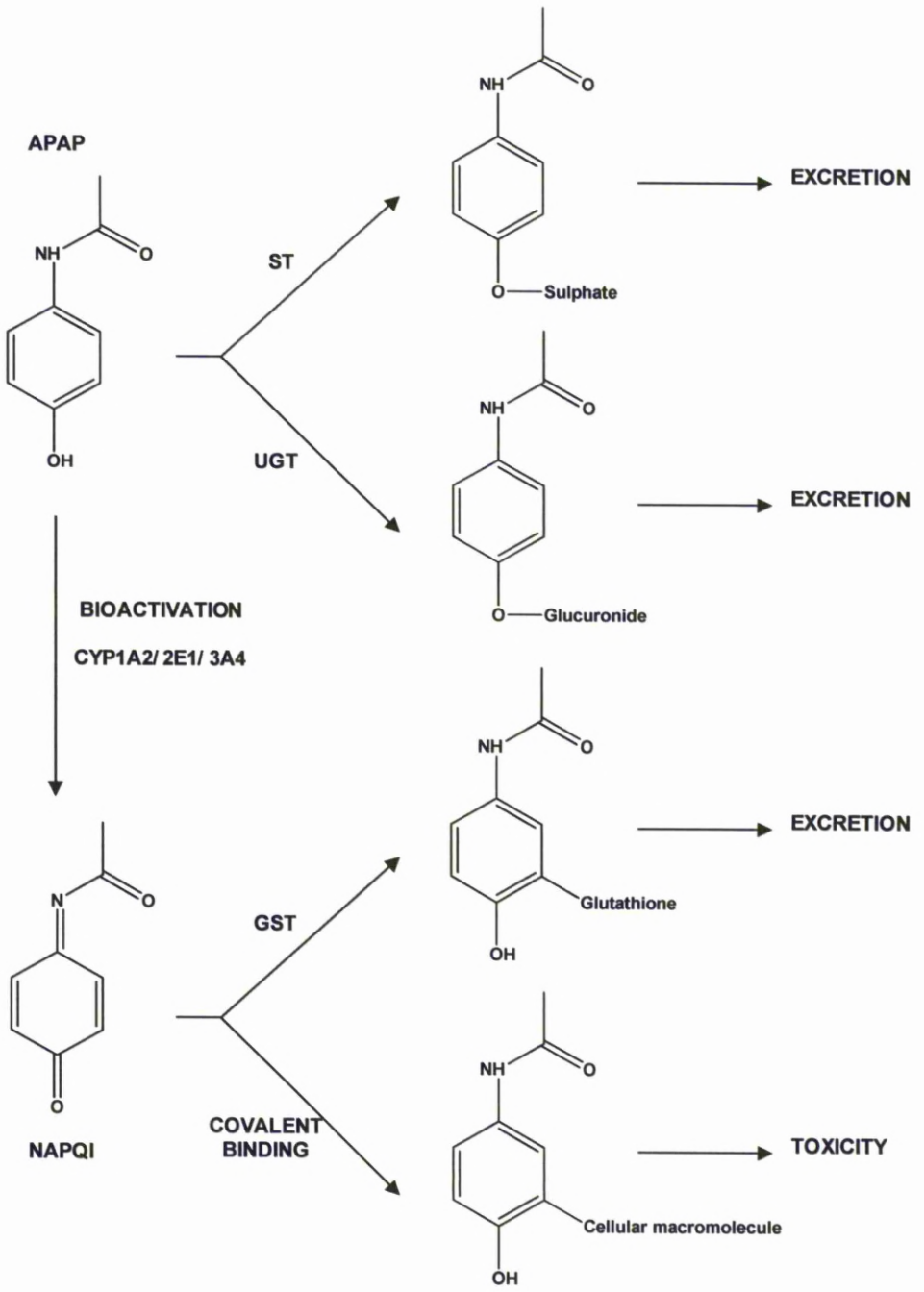

Figure 1.1: Metabolism and toxicity of APAP.
At therapeutic doses APAP is detoxified to glucuronide and sulphate metabolites, with a small proportion (5-10%) bioactivated to the electrophilic reactive metabolite NAPQI, which is then conjugated with GSH and detoxified. During overdose, the glucuronidation and sulphation pathways become saturated leading to increased formation of NAPQI. Intracellular stores of GSH are depleted leading to an accumulation of NAPQI, which can lead to covalent binding of cellular macromolecules and toxicity.

1.4.2 Cellular defence in response to APAP bioactivation and bioinactivation

The depletion of hepatic GSH enables NAPQI to covalently modify and inhibit the function of various critical proteins within the hepatocyte, which can lead to cellular dysfunction and cell death. The covalent modification or functional inhibition of critical proteins such as γ-glutamyl cysteine ligase, catalytic subunit (GCLC) (Kitteringham et al., 2000), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (Dietze et al., 1997) and Ca^{2+}/Mg^{2+} ATPase (Tsokos-Kuhn et al., 1988) can severely impair hepatocyte function. The covalent modification of these proteins by NAPQI has been hypothesised to contribute to mitochondrial dysfunction and disruption of ATP and Ca^{2+} homeostasis (Jaeschke et al., 2003a) and is thought to be a critical step in the development of cell death observed following APAP overdose.

One of the major tiers of cell defence involves the upregulated expression of cytoprotective genes, a process mediated by certain transcription factors. In addition to inducing direct cellular damage, oxidants can activate transcription factors, which regulate the production of inflammatory mediators (Dambach, 2006). The massive chemical stress mediated by an APAP overdose leads to an immediate adaptive defence response in the hepatocyte. This involves various mechanisms, including the nuclear translocation of redox-sensitive transcription factors such as nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf-2), which 'sense' chemical danger and orchestrate cell defence (Goldring et al., 2004). Nrf2 is essential for the antioxidant responsive element (ARE)-mediated induction of phase II detoxifying and oxidative stress enzyme genes (Itoh et al., 1999). In the absence of cellular stress the cytoplasmic protein kelch-like ECH-associated protein 1 (Keap1) directly binds to Nrf2 and represses transactivation by promoting proteasome-dependent degradation of the protein. Exposure to oxidative or chemical stress enables Nrf2 to evade the Keap1-mediated degradation process, and accumulates within the nucleus to activate ARE target genes (Itoh et al., 1999; Goldring et al., 2004) (Figure 1.2). With respect to APAP, Nrf2 genes of immediate significance are those involved in glutathione synthesis such as GCLC, GSTs, glucuronyltransferases, and heme oxygenase (OH). Importantly, it has been observed that nuclear translocation occurs at nontoxic doses of APAP and at time-points before overt toxicity is observed. However, with increasing doses of APAP, there is progressive dislocation of nuclear translocation, transcription, translation, and protein activity

(Kitteringham et al., 2000) as the rate of drug bioactivation overwhelms cell defence through the modification and inhibition of critical proteins.

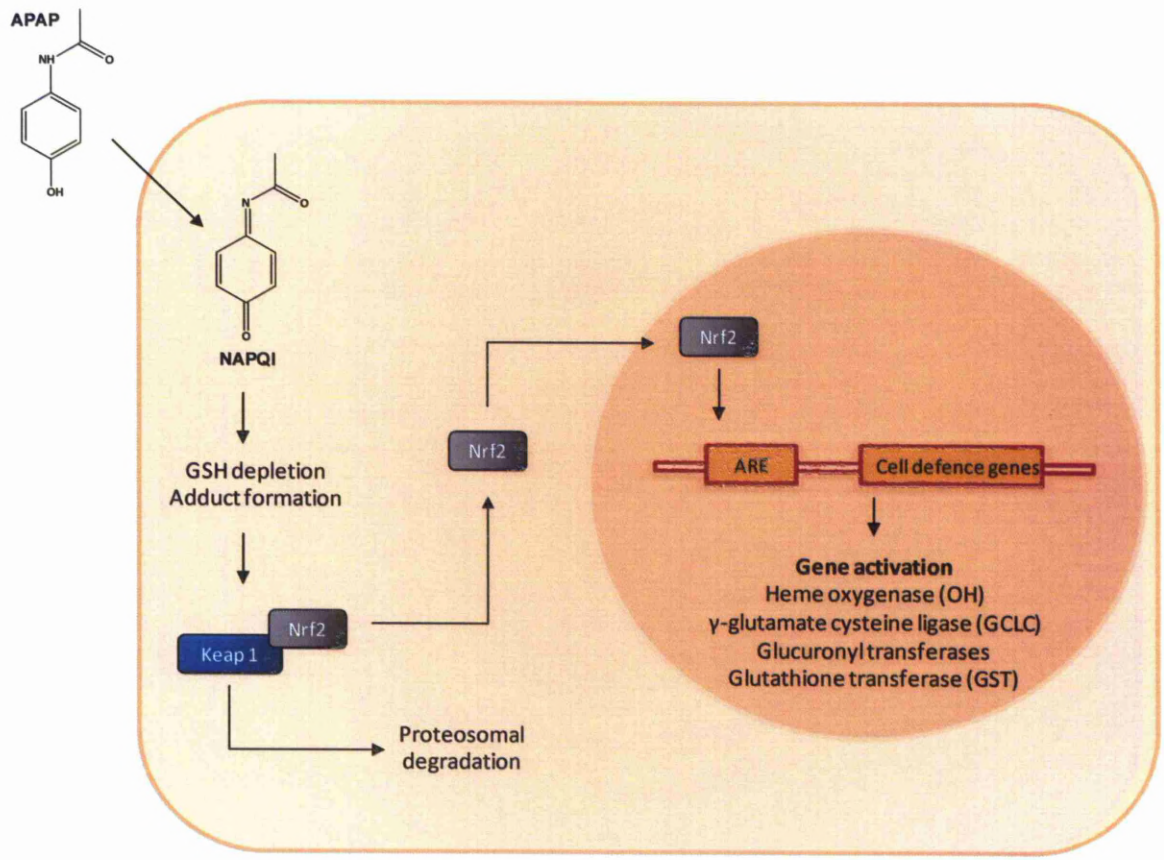

Figure 1.2: Cellular defence by Nrf2-ARE pathway in response to APAP.
Formation of NAPQI in hepatocytes results in GSH depletion and adduct formation. This leads to release of Nrf2 from its cytoplasmic inhibitor, Keap 1, which then translocates to the nucleus. Nrf2 in the nucleus activates the antioxidant response element (ARE), resulting in the enhanced transcription of a variety of genes involved in cell defence (adapted from Park et al., 2005).

Nuclear factor-κB (NF-κB) is the name given to a family of transcription factors formed by the hetero- or homodimerisation of proteins from the Rel family (Lawrence et al., 2001). There are 5 Rel proteins: p50, p65 (RelA), c-Rel, p52, and RelB (Hayden et al., 2006). NF-κB is a major regulator of the innate and adaptive immune response, and thus serves an important role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and

inducible enzymes which include cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In resting cells, NF-κB is localised within the cytosol as a dimeric complex, usually comprising p50 and p65 subunits (Hayden et al., 2006). The cytoplasmic NF-κB exists in an inactive form due to binding to an inhibitor protein, IκB (Baeuerle and Baltimore., 1988; Li et al., 2002). NF- κB activation is mediated by a number of cell surface receptors, including TNF-α receptor, interleukin-1 receptor, toll-like receptors (TLRs) and receptor for advanced glycation end products (RAGE). Activation of NF-κB by stimulation of the cell through these receptors in response to a variety of stimuli such as reactive oxygen species (ROS), the bacterial endotoxin lipopolysaccharide (LPS) or proinflammatory cytokines, is a result of the phosphorylation of IκB, which targets the molecule for ubiquitination and then subsequent proteosomal degradation (Hayden et al., 2004). Phosphorylation of IκB is mediated by an enzyme complex called IκB kinase (IKK), which phosphorylates critical serine residues within IκB. IKK is a dimer consisting of two different subunits, IKKα and IKKβ, which is held in an IKK complex with a regulatory subunit called NF-κB essential modifier (NEMO) (Scherer et al. 1995; Mercurio et al. 1997; Mercurio et al. 1999; Ben-Neriah 2002). Consequently, the nuclear localisation signal (NLS) of NF-κB is revealed, permitting translocation of NF-κB into the nucleus, where it can bind to the promoter region of various target genes (Figure 1.3). NF-κB is activated during APAP-induced hepatotoxicity (Blazka et al., 1996a, 1996b; Bauer et al., 2000; Chiu et al., 2003a, 2003b) suggesting that transcription factors may be involved in the inflammatory response to this hepatotoxicant. Notably, exposure of mice to hepatotoxic doses of APAP causes the NF-κB-dependent upregulation of pro-inflammatory mediators, including IL-1β and TNFα, and the anti-inflammatory cytokine IL-10 in the liver (Dambach et al. 2006).

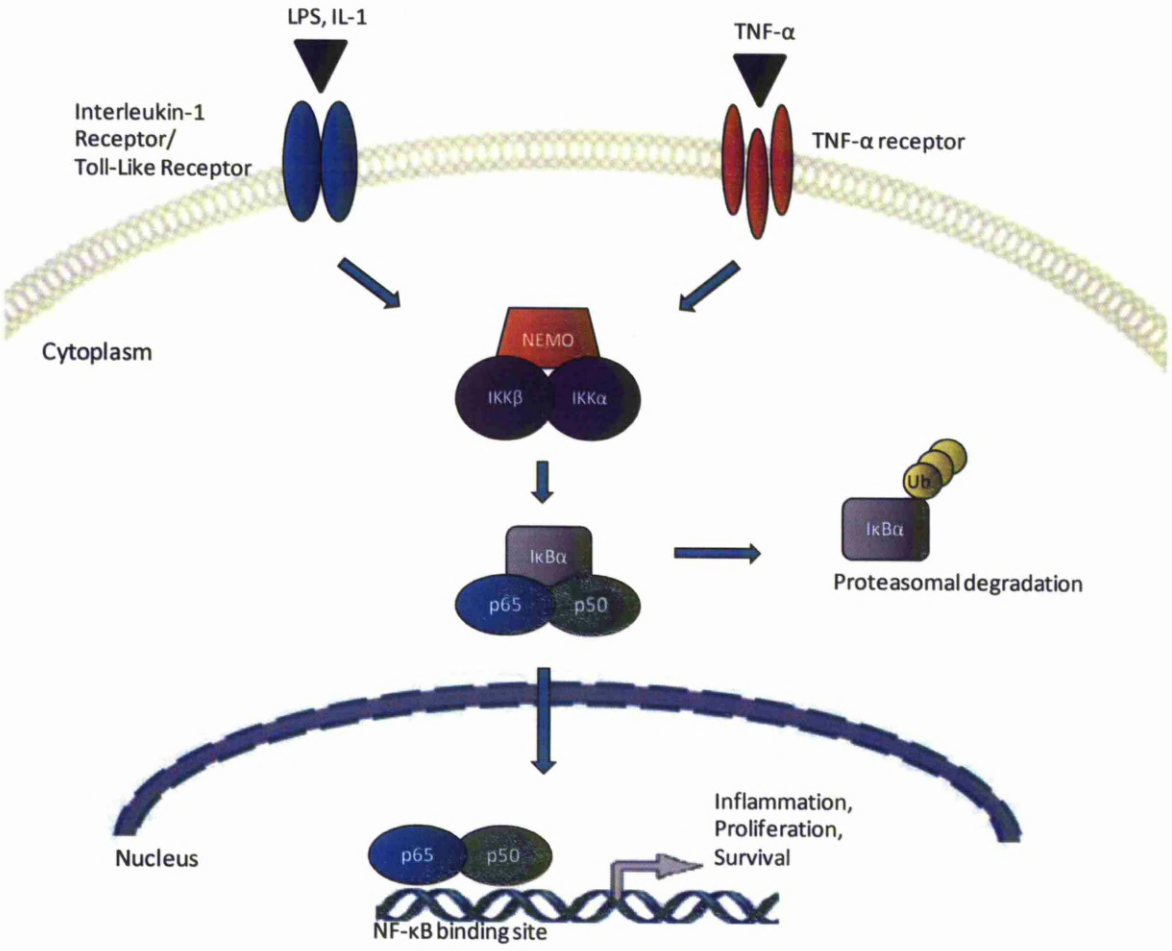

Figure 1.3: NF-κB signal transduction pathway.
In the absence of inflammatory activity, the transcription factor NF-κB is retained in the cytoplasm by the protein inhibitor IκB. Proinflammatory stimuli such as LPS, IL-1 or TNF-α activate the protein kinase complex IKK, resulting in the degradation of IκB and translocation of NF-κB into the nucleus (adapted from Perkins 2007).

1.4.3 APAP-induced hepatic cell death

APAP has been shown to result in necrotic hepatocyte cell death (Jaeschke et al., 2003a) and it is generally accepted that necrosis is the final and ultimate form of hepatic cell death in APAP-induced hepatotoxicity in animals and in man. However, some reports suggest hepatic apoptosis can also occur following APAP metabolic activation (Ferret et al., 2001; Henderson et al., 2007; Kon et al., 2007) and may even play a key role in modulation of APAP

hepatotoxicity (Ray et al., 1996; El-Hassan et al., 2003). Despite intense research, the role of apoptosis remains controversial with some reports suggesting that apoptosis may not feature in APAP hepatotoxicity due to overwhelming oxidative stress and rapid depletion of ATP in the hepatocyte, conditions which are not favourable for caspase activation, a prerequisite for apoptosis (Lawson et al., 1999; Ruepp et al., 2002; Cover et al., 2005; Gunawan et al., 2006).

1.4.4 Effect of fasting on APAP-induced hepatotoxicity

As mentioned previously, GSH depletion is known to be a prerequisite for the onset of toxicity (Mitchel et al., 1973b). Fasting has been shown to substantially reduce intracellular hepatic GSH levels, resulting in a markedly increased susceptibility to the toxic effects of APAP (Pessayre et al., 1979). A reduced hepatic content of GSH, required to detoxify the reactive metabolite, has been documented in chronic alcoholics and has been postulated to be a factor responsible for the liver injury described in alcoholic patients following therapeutic doses of APAP (McClain et al., 1980; Jewell et al., 1986; Seeff et al., 1986). Malnourished individuals may therefore have a compromised detoxification capacity due to decreased levels of hepatic GSH and become more susceptible to APAP hepatotoxicity.

1.5 INNATE IMMUNOLOGICAL RESPONSE TO APAP HEPATOTOXICITY

1.5.1 Sterile inflammation and damage-associated molecular pattern molecules

Inflammation is the first line of response to tissue damage. Blood flow is increased, and both nucleated blood cells and fluid extravasate from the vessels into the affected tissue. Neutrophils, followed later by macrophages are recruited to provide a first line of defence against intruding microorganisms, and to clear the debris (Ramaiah et al., 2007). Inflammation is considered a dual process since it can have two functionally different characteristics, one that can lead to tissue regeneration and another that can produce damage. Inflammation is part of a physiological process for repairing damage, however, when this process is not controlled and inflammation spreads, it loses its repairing and regenerating function and which may cause damage (Nathan, 2002; Aller et al., 2006). Molecules

generated to kill microbes, such as reactive oxygen species and proteases, leak from live and dying leukocytes and kill normal cells (Rock et al., 2008). Recent studies have shown that dying cells trigger inflammation even in the absence of microbial infection, known as sterile inflammation (Chen et al., 2007). Sterile inflammation occurs as a result of recognition of intracellular proteins released by necrotic cells, known as alarmins or damage-associated molecular patterns (DAMPs) which results in the recruitment of neutrophils. DAMPs are host-derived proteins that are released following tissue injury or cell death and have the ability to activate a proinflammatory response. These include heat-shock proteins (HSPs) (Vabulas et al., 2001; Quintana et al., 2005), DNA (Jahr et al., 2001), ATP (Mariathasan et al., 2006), and high mobility group box-1 (HMGB1) (Hori et al., 1995; Yu et al., 2006; Tian et al., 2007). Under normal physiological conditions, DAMPs are usually sequestered intracellularly and therefore hidden from recognition by cells of the innate immune system, however when released into the extracellular environment by necrotic cells they can trigger an immune response (Rubartelli et al., 2007). One of the DAMPs identified to be released during APAP hepatotoxicity was HMGB1 and treatment of mice with HMGB1-specific antibodies ameliorated hepatic inflammatory cell recruitment (Scaffidi et al., 2002).

In summary, during an inflammatory response induced by released cellular contents, the tissue site is rapidly infiltrated by leukocytes, consisting initially of neutrophils followed by accumulations of macrophages and production of proinflammatory cytokines and chemokines. There is an increasing awareness that during APAP hepatotoxicity, nonparenchymal cells of the liver and cells of the innate immune system, namely neutrophils, Kupffer cells and natural killer/natural killer with T cells (NK/NKT cells) may be involved in the pathogenesis (Jaeschke et al., 2005; Liu et al., 2006) (Figure 1.4). The use of gene knock-out animals (Ishida et al., 2002; Bourdi et al., 2007) and the depletion of certain cell types (Ju et al., 2002; Liu et al., 2004; Liu et al., 2006) have highlighted the critical role played by the innate immune system in the modulation of hepatotoxicity.

1.5.2 Role of neutrophils in APAP hepatotoxicity

APAP-induced liver injury has been histopathologically characterised by centrilobular necrosis and infiltration of leukocytes, mainly polymorphonuclear leukocytes (neutrophils).

Hepatic infiltration and activation of neutrophils is an early response to tissue injury, cellular stress or systemic inflammation and is a hallmark of the inflammatory process, which may contribute to the development of APAP-induced liver injury (Luster et al., 2001; Ishida et al., 2006; Jaeschke et al., 2006). Increasing evidence has implicated neutrophils as the main type of leukocyte involved in the pathogenesis of APAP-induced liver injury (Jaeschke et al., 2002; Liu et al., 2006). A study showed that depletion of neutrophils using an anti-Gr-1 antibody significantly protected mice against APAP-induced liver injury, as evidenced by markedly reduced serum ALT levels and centrilobular hepatic necrosis, and improved mouse survival (Liu et al., 2006). Another study demonstrated that the magnitude of liver injury induced by APAP correlated with hepatic neutrophil number and myeloperoxidase activity, suggesting that neutrophil recruitment in the liver was involved in the pathogenesis of APAP-induced liver injury (Ishida et al., 2002). The authors went on in a more recent study to show that neutrophils expressed CXCR2 and inducible NO synthase (iNOS), which can produce NO, a potent injurious mediator for APAP-induced liver injury (Ishida et al., 2006). There are however, conflicting views concerning the role of neutrophils in APAP-induced liver injury (Lawson et al., 2000). One study suggested that although the numbers of neutrophils were increased in the liver of APAP-challenged animals, neutrophils did not contribute to the initiation and progression of APAP-induced liver injury. The inflammation observed after overdose was stated as not being severe enough to cause additional damage (Lawson et al., 2000). Enhanced Mac-1 (CD11b/CD18) expression and L-selectin (CD62L) shedding are characteristic for activation of neutrophils (Jutila et al., 1989). However, it was shown that there was neither Mac-1 upregulation nor L-selectin shedding of circulating neutrophils after APAP administration suggesting that there was no activation of neutrophils during APAP-induced toxicity (Lawson et al., 2000).

1.5.3 Role of Kupffer cells in APAP hepatotoxicity

Kupffer cells are resident macrophages of the liver and constitute 80-90% of all tissue-resident macrophages in the body, representing 15% of the total liver cell population (Bouwens et al., 1986). Predominantly, they are localised in the lumen of the hepatic sinusoids in the periportal and centrilobular regions of the liver lobule (Bouwens et al., 1986). They play an important role in the normal physiology and homeostasis of the liver, but can also participate in the acute and chronic responses of the liver to toxic compounds. The

activation of Kupffer cells has been previously attributed to APAP hepatotoxicity (Laskin et al., 1986; Michael et al., 1999; Ito et al., 2003) by the release of an array of cytokines and reactive oxygen species. This was evident in a study that used gadolinium chloride ($GdCL_3$), which selectively reduces the capacity of Kupffer cells to produce superoxide (Liu et al., 1995). Pretreatment of mice with $GdCL_3$ completely eliminated oxidative stress and liver injury during the first 8hr post APAP-dose (Michael et al., 1999). However, subsequent studies using $GdCL_3$ found that little to no protection was afforded against APAP hepatotoxicity (Ju et al., 2002; Ito et al., 2003; Knight et al., 2004). Moreover, another report contradicted the theory of hepatotoxicity potentiation by Kupffer cells with the authors demonstrating a hepatoprotective role (Ju et al., 2002). This was highlighted through the near complete depletion of Kupffer cells, using liposome-entrapped clodronate, resulting in increased susceptibility of mice to APAP hepatotoxicity. This was thought to be a result of the Kupffer cell depletion leading to the elimination of the formation of several hepatoregulatory cytokines and mediators including IL-6 and IL-10. The conclusion was that the dominant effect of Kupffer cell activation after APAP is not to cause cytotoxicity by oxidant formation but rather to limit toxicity by production of a number of hepatoregulators that serve to potentially counteract proinflammatory stimuli and support liver regeneration. The discrepancies between reports are based on the use of different agents to deplete or inhibit Kupffer cell function (Ju et al., 2002) and serve to highlight the complexity of the role Kupffer cells play in APAP-induced liver injury.

1.5.4 Role of natural killer and natural killer T cells in APAP hepatotoxicity

It was previously reported that the depletion of natural killer (NK) cells and NK cells with T-cell receptors (NKT cells) in the liver had a protective effect against APAP hepatotoxicity (Liu et al., 2004). In this study, mice were depleted of both NK and NKT cells by an anti-NK1.1 antibody and treated with 500mg/kg APAP. The authors found that the increase in a large number of cytokines and chemokines including interferon-γ (IFN-γ) and monocyte chemoattractant protein-1 (MCP-1) after APAP treatment was significantly reduced. Also, they observed a decrease in Fas ligand (FasL) expression and a reduction in neutrophil accumulation in the liver. The effect of APAP on cytokines, chemokines, FasL expression and neutrophil accumulation was also reproduced in IFN-γ deficient mice (Ishida et al., 2002;

Liu et al., 2004). It was concluded that NK and NKT cells were mainly responsible for IFN-γ production and the subsequent severity of liver injury after APAP (Liu et al., 2004). However, it was later implicated in a conflicting report that the use of dimethyl sulfoxide (DMSO) to solubilise APAP was the major factor in the contribution of NKT and NK cells to the overall extent of APAP-induced liver injury (Masson et al., 2008).

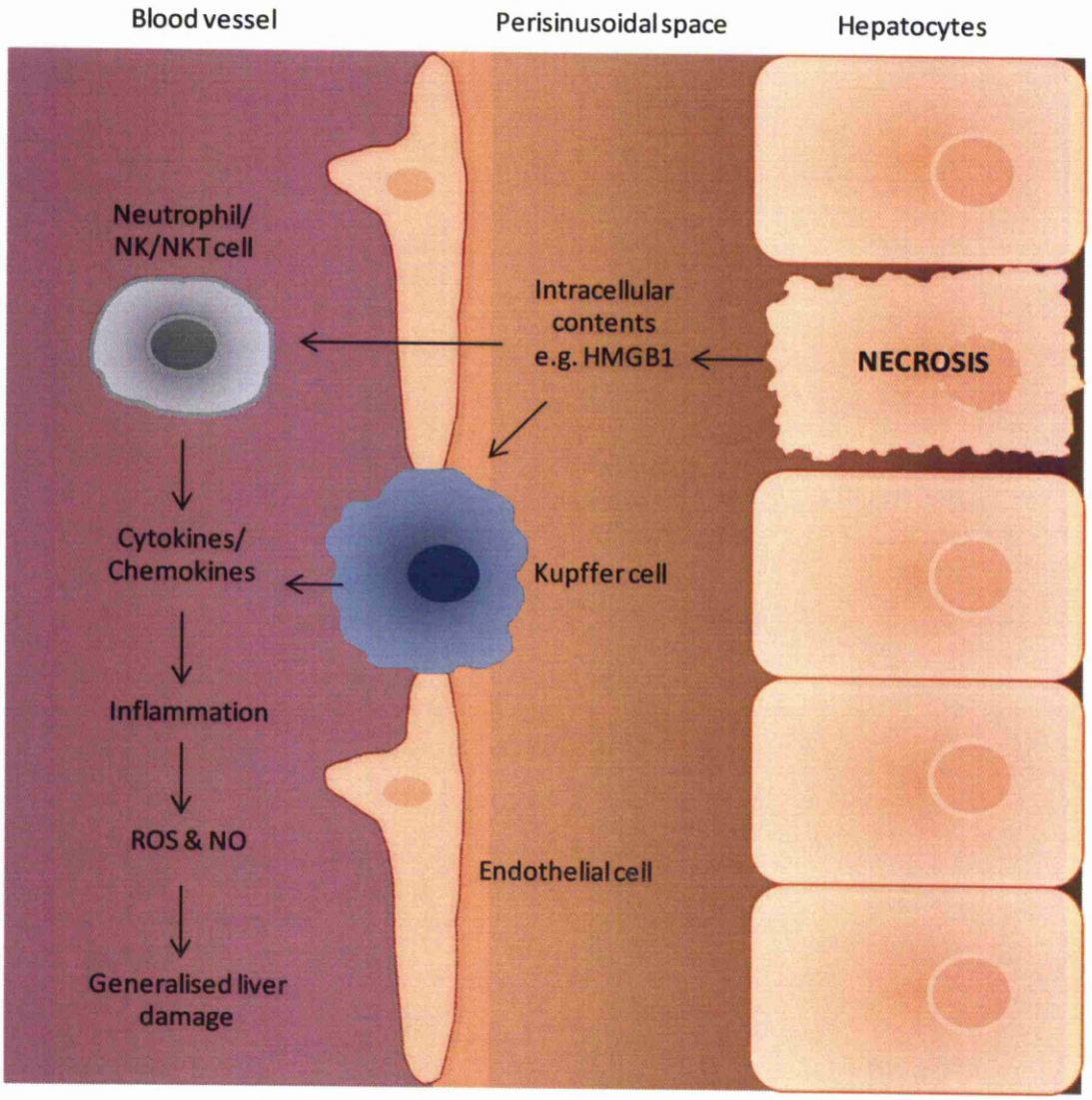

Figure 1.4: Overview of the effect of the release of intracellular contents from necrotic hepatocytes and activated immune cells in the modulation of DILI.
Necrotic hepatocytes passively release HMGB1 into the extracellular space and blood. Activation of innate immune cells (Kupffer cells, neutrophils and NK/NKT cells) by HMGB1 and other DAMPS triggers the further release of HMGB1 and inflammatory cytokines, therefore modulating the overall pathogenesis of DILI (adapted from Adams et al., 2010).

1.6 CIRCULATING MEDIATORS OF INFLAMMATION INVOLVED IN DILI

In addition to the intracellular biochemical events there is growing evidence that suggests that the innate immune system can contribute to the severity and progression of APAP-induced liver injury through the recruitment of inflammatory cells into the liver and production of cytokines downstream of bioactivation (Blazka et al., 1995; Laskin et al., 2001; Liu et al., 2004). The generation of inflammatory mediators such as cytokines, chemokines, reactive oxygen and nitrogen species released by Kupffer cells and macrophages have been implicated in APAP hepatotoxicity by exacerbating cell damage as a result of initiating an overly aggressive inflammatory process (Luster et al., 2001; Liu et al., 2004). Cytokines are small, soluble protein mediators produced by mostly all cell types in response to various stimuli including other cytokines. They play important roles in the normal physiology of cells and are commonly associated with the immune response, inflammation, tissue injury or repair, and organ dysfunction (Simpson et al., 1997; DeCicco et al., 1998). Many cytokines, such as TNF-α, IL-1β and IL-6, have been implicated as key mediators of APAP hepatotoxicity. An understanding of the role of cytokine responses in APAP hepatotoxicity is therefore important to the future development of novel therapies for APAP toxicity.

1.6.1 Tissue necrosis factor-α (TNF-α)

TNF-α is a proinflammatory cytokine that aids in tissue regeneration by stimulating apoptosis and cell proliferation but also can exacerbate cell damage by initiating an overly aggressive inflammatory response. It is produced by a number of different cells in the liver, including Kupffer cells (Decker et al., 1990), endothelial cells (Ranta et al., 1999) and hepatocytes (Gonzalez-Amaro et al., 1994). Production of TNF-α is an early event in a hepatic inflammatory response and triggers the release of a cascade of cytokines that recruit and activate inflammatory cells (Bradham et al., 1998; Luster et al., 2001). TNF-α has been shown to increase inflammatory cell infiltration by promoting recruitment and adhesion of neutrophils to endothelial cells (Fiers., 1991). The biological effects of TNF-α are mediated by two receptors, TNFR1 and TNFR2, both of which are structurally related but are involved in different distinct signalling pathways (Tartaglia et al., 1991; Tartaglia et al., 1993; Bradham et al., 1998). The receptors are co-expressed on the surface of many cell types with

TNFR1 being heavily expressed on Kupffer cells and hepatocytes (Faubion and Gores., 1999). TNF-α signalling via TNFR1 is the main signalling pathway leading to an inflammatory response (Douni et al., 1998; Peschon et al., 1998). TNF-α has been implicated to have a role in the exacerbation of APAP hepatotoxicity as it was shown to be produced after APAP challenge and was responsible for the progression of APAP hepatotoxicity (Blazka et al., 1995; Blazka et al., 1996; Goldin et al., 1996). It was demonstrated that TNF-α is generated between 4-24hr after APAP overdose, and the administration of antibodies targeted against TNF-α attenuated APAP-induced liver injury (Blazka et al., 1995). In contrast, one study showed that antibodies to TNF-α had no effect on liver damage induced by APAP (Simpson et al., 2000). Similarly, no difference in APAP-induced liver injury was observed between TNF-α KO mice and wild-type mice (Boess et al., 1998). The differences between these reports were suggested to be due to the doses of APAP used and the sensitivities of different mouse strains used in these studies (Gardner et al., 2003). Due to the dual role played by TNF-α in tissue repair and tissue damage it appears that its role in APAP hepatotoxicity depends on the balance and kinetics of TNF-α production as well as other mediators in the liver (Gardner et al., 2003).

1.6.2 Interleukin 6 (IL-6)

IL-6 is a multifunctional cytokine that has pro- and anti-inflammatory properties that regulate the immune response, haematopoiesis, the acute-phase response and inflammation (Hirano et al., 1998; Heinrich et al., 2003). Il-6 has been shown to have a pathogenic proinflammatory role in animal models of experimental autoimmune diseases such as insulin-dependent diabetes mellitus, inflammatory bowel disease and rheumatoid arthritis (Ishihara et al., 2002). In contrast, IL-6 has been shown to protect against liver injury caused by carbon tetrachloride (CCl4) (Kovalovich et al., 2000), ethanol (Hong et al., 2002), ischaemia/reperfusion (Camargo et al., 1997) and APAP (James et al., 2003; Masubuchi et al., 2003). Studies have reported the upregulation of IL-6 in animal models of hepatic injury (Luckey et al., 2002) including APAP treated mice (Bourdi et al., 2002a; James et al., 2003). Other investigations using IL-6 KO mice suggest that IL-6 is important in hepatocyte regeneration following APAP hepatotoxicity (James et al., 2003; Masubuchi et al., 2003). In these studies it was demonstrated that in the absence of IL-6 in APAP treated mice, toxicity was increased compared to wild-type mice. The pro- and anti-inflammatory role of IL-6 in APAP

hepatotoxicity may be determined by the level of expression of the cytokine as one report suggested that IL-6 can be a protoxicant in a model of APAP-induced liver injury when it is overexpressed (Bourdi et al, 2002a). This study demonstrated that using IL-10/IL-4 KO mice, IL-6 expression levels were elevated and mice were highly susceptible to APAP hepatotoxicity. The enhanced sensitivity of these mice to APAP was diminished when IL-6 levels were reduced using an anti-IL-6 antibody. They concluded that the deficiency of IL-10 and IL-4, which downregulates IL-6, lead to high levels of IL-6 and subsequent sensitisation of mice to APAP hepatotoxicity.

1.6.3 Interleukin 1 (IL-1α/IL-1β)

There are two distinct forms of IL-1, IL-1α and IL-1β, both of which are pleiotropic cytokines that mediate a range of biological activities such as proinflammatory responses, the immune response and haematopoiesis (Dinarello et al., 1991). IL-1α and IL-1β stimulate the IL-1 receptor (IL-1R) which itself has different types. The type I IL-1R is a transmembrane protein that is expressed predominantly by T cells, fibroblasts, and endothelial cells. IL-1R type II is a transmembrane protein found on B lymphocytes, neutrophils, monocytes, large granular leukocytes and endothelial cells (Kuno et al., 1994). IL-1 has been implicated in the injury process of APAP toxicity in experimental animals. In one report, IL-1R KO mice were more than 90% protected against APAP hepatotoxicity based on plasma ALT activities (Chen et al., 2007). However, when a combination of neutralising antibodies against IL-1R, IL-1α and IL-1β was used, the protective effect was reduced to 30%. An earlier study also indicated that antibodies against IL-1α protect against APAP hepatotoxicity in mice (Blazka et al., 1995; Blazka et al., 1996).

1.6.4 High mobility group box protein 1 (HMGB1)

The chromosomal protein HMGB1 is a member of the high mobility group box family (HMGB; previously known as amphoterin or HMG), which include HMGB2 and HMGB3 (Muller et al., 2001; Degryse et al., 2003; Lotze et al., 2005). HMGB1 was first identified over 30 years ago and later determined as an abundant nonhistone protein present in nuclei and cytoplasm of almost all eukaryotic cells (Goodwin et al., 1973; Muller et al., 2004). Its

main intranuclear function is to modulate chromatin structure to facilitate transcription (Bianchi et al., 2004). HMGB1 is a 25kDa protein whose structure is composed of two homologous DNA-binding domains (Box A and B), and a negatively charged C-terminal domain made up of a carboxylic acid tail composed of aspartic and glutamic acids (Bonaldi et al., 2003) (Figure 1.5). The acidic tail has important functional roles such as the modulation of the acetylation of HMGB1 by histone acetyltransferases (Pasheva et al., 2004).

The nuclear role of HMGB1 has been widely studied. It has been found to be loosely associated with chromatin of cells and participates in DNA replication, gene transcription and repair as well as the regulation of chromatin structure (Chen et al., 2004; Yang et al., 2005). The change in structure enables multiple interactions between DNA and proteins such as p53, NF-κB and steroid hormone receptors (Bianchi et al., 2007). The functional importance of HMGB1 as a regulator of gene transcription has been determined by HMGB1 knockout mice, which die shortly after birth due to hypoglycaemia and show a defect of the transcriptional function of the glucocorticoid receptor (Calogero et al., 1999; Knapp et al., 2004). Expression of the recombinant B box has been highlighted as the domain responsible for the proinflammatory cytokine function, inducing the release of IL-6 and TNF-α whereas the recombinant A box acts as a specific antagonist by attenuating HMGB1-induced macrophage secretion of these proinflammatory cytokines (Yang et al., 2002; Li et al., 2003). HMGB1 undergoes post-translational modifications including phosphorylation, acetylation, methylation (Bianchi et al., 2007) and oxidation (Hoppe et al., 2006). The processes leading to these modifications and their functional consequences remain largely unknown. Post-translational modifications of HMGB1 appear to be important for its subcellular localisation and may regulate its proinflammatory activity (Lotze et al., 2005).

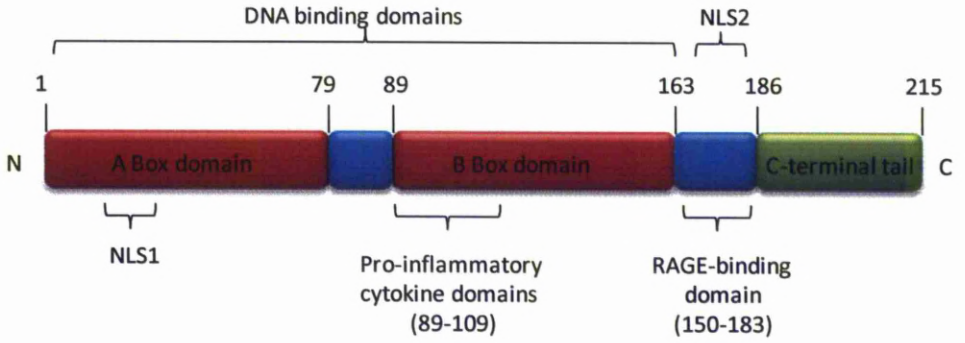

Figure 1.5: Schematic representation of the structural domains of HMGB1.
HMGB1 is a tripartite structure composed of two homologous DNA-binding domains (named A-box and B-box), and a negatively charged acidic C-terminus. The two nuclear localisation signal sequences (NLS) undergo acetylation and are involved in nuclear transport. HMGB1 can activate the receptor of advanced glycation end products (RAGE) through the RAGE-binding domain located at the amino acids 150–183 (adapted from Ulloa and Messmer., 2006).

HMGB1 has been shown to play an important pathogenic role in a wide range of inflammatory diseases such as sepsis (Andersson et al., 2003; Sunden-Cullberg et al., 2005), rheumatoid arthritis (Taniguchi et al., 2003), inflammatory lung disease (Abraham et al., 2000) and systemic lupus erythematosus (SLE) (Urbonaviciute et al., 2008). HMGB1 has been identified as a late mediator of endotoxin lethality as murine and human macrophages/monocytes can release large amounts of HMGB1 days after being stimulated by exposure of the bacterial endotoxin, lipopolysaccharide (LPS) (Wang et al., 1999). HMGB1 is secreted from monocytes/macrophages within 16-36hr following stimulation with LPS, TNF-α, or IL-1β, which is in contrast to the secretion of the proinflammatory cytokines TNF-α and IL-1β which is within minutes of LPS stimulation both *in vitro* and *in vivo* (Wang et al., 1999; Andersson et al., 2000; Bianchi et al., 2007). Antibodies directed towards neutralising HMGB1 have shown to protect against LPS-induced lethality even when administered 24 hours after LPS stimulation.

HMGB1 can be actively secreted by stimulated immune cells or by passive release during necrotic cell death but not by apoptosis (Scaffidi et al., 2002; Rovere-Querini et al., 2004) HMGB1 is released in a delayed manner from activated macrophages or monocytes compared with the earlier production and secretion of TNF-α and IL-1 in response to

stimulation with LPS, pro-inflammatory cytokines such as TNF-α, IL-1β, IFN-γ and other signalling molecules (Wang et al., 1999; Chen et al., 2004; Erlandsson Harris, 2004). HMGB1 is relocalised from the nucleus to cytoplasmic organelles after stimulation by LPS or pro-inflammatory cytokines (Gardella et al., 2002). In most cells, HMGB1 is continuously shuttled between the nucleus and the cytoplasm due to two non-classical nuclear export signals (NES1 and NES2) but nuclear accumulation is favoured (Bonaldi et al., 2003). It was discovered that activation of macrophages/monocytes as a result of LPS stimulation, or forced hyperacetylation by acetylases, leads to acetylation of two specific clusters of lysine residues on HMGB1 that causes it to accumulate in the cytoplasm as its nuclear re-entry is blocked but not its nuclear export (Bonaldi et al., 2003; Bianchi et al., 2004; Wahamaa et al., 2007). Moreover, once the HMGB1 is cytosolic, it is moved further by accumulating in secretory lysosomes which are specialised organelles that are abundant in haematopoietic cells (Bonaldi et al., 2003). The HMGB1-containing secretory lysosomes can then fuse with the cell membrane and exocytosis is induced by cellular activation by lysophosphatidylcholine (LPC), a bioactive lipid generated by phospholipase A2 that promotes inflammatory effects (Gardella et al., 2002). The release of HMGB1 for its role as an inflammatory cytokine is not solely dependent on activated inflammatory cells as it can also be released by necrotic cells in a hypo-acetylated form. Cells that undergo necrosis or are damaged lose the integrity of their membranes, which causes their soluble intracellular contents such as proteins to leak out (Bianchi et al., 2004). HMGB1 is passively released from necrotic cells as a soluble molecule which is capable of inducing inflammatory responses both *in vivo* and *in vitro* (Scaffidi et al., 2002; Dumitriu et al., 2005). Wild-type necrotic cells promoted a strong inflammatory response from macrophages whereas HMGB1 null necrotic cells had a reduced ability to signal inflammation. HMGB1 might be a critical molecule that allows innate immune cells both to respond to injury, and to further induce inflammation.

This is in contrast to apoptotic cells, which do not release significant quantities of HMGB1, as these cells retain HMGB1 in their nuclei, which become firmly bound to chromatin, due in part to chromatin hypoacetylation (Scaffidi et al., 2002). The mechanism in which HMGB1 is retained in apoptotic cells was shown to involve the underacetylation of histones, with the affinity of HMGB1 for DNA to be higher with a lower degree of acetylation. If apoptotic cells are prevented from modifying their chromatin, HMGB1 will no longer be retained and

the cells become as inflammatory as necrotic cells (Scaffidi et al., 2002). This difference between the types of cell death might underlie important differences in the ability of apoptotic and necrotic cells to promote an inflammatory response (Lotze et al., 2005). Having two distinct mechanisms of release demonstrates that HMGB1 can act as an early initiator (by means of passive release from necrotic cells) and a late promoter (by means of the late, active release by activated macrophages/monocytes) of inflammation (Wang et al., 1999; Erlandsson Harris, 2004).

Once released, HMGB1 targets innate immune cells through Toll-like receptors (TLR) and the receptor for advanced glycation end-products (RAGE) (Stern et al., 2002; Yu et al., 2006; Palumbo et al., 2007). Binding of HMGB1 to RAGE, TLR2 and 4 can lead to recruitment of inflammatory cells by acting as a chemoattractant and secretion of proinflammatory cytokines, therefore having the potential to modulate the overall extent of liver injury. Interaction of HMGB1 with these receptors could provide a critical link between tissue damage and activation of the innate immune response and provide a novel area of therapeutic intervention for the treatment of DILI. Antibodies directed towards neutralising HMGB1 have been shown to be successful in attenuating the inflammatory response associated with APAP hepatotoxicity (Scaffidi et al., 2002) and LPS-induced lethality in mice (Wang et al., 1999). Administration of ethyl pyruvate also was shown to attenuate systemic inflammatory responses and improve survival in mice subjected to lethal peritonitis by inhibiting HMGB1 release from activated immune cells (Ulloa et al., 2002). As well as inhibiting HMGB1, targeting its receptors may also help reduce the inflammatory response associated with APAP hepatotoxicity.

1.7 RECEPTOR MEDIATORS OF INFLAMMATION

1.7.1 Toll-Like receptors (TLRs)

Toll-like receptors (TLRs) are highly conserved proteins that recognise specific molecular patterns that are present in a variety of endogenous and exogenous stimuli and play a crucial role in the induction of an immune response (Aderem et al., 2000). TLR signalling can be

activated by molecules released by injured tissue, namely damage-associated molecular patterns (DAMPs), which include HMGB1. TLR proteins transduce signals through a family of cytosolic adapter proteins such as MyD88. After receptor engagement, MyD88 recruits members of the death domain-containing serine/threonine IL-1R-associated kinase (IRAK) family which leads to the activation and nuclear translocation of NF-κB (Park et al., 2004; Yu et al., 2006). Toll-like receptor 2 and 4 (TLR2 and TLR4) have been implicated in HMGB1 signalling in neutrophils and macrophages (Park et al., 2004; Yu et al., 2006). Recent findings implicated the involvement of TLR2 and TLR4 in HMGB1 signalling using transfection experiments of murine macrophage cell lines with dominant negative constructs of the toll-like receptors (Park et al., 2004). The macrophage cell lines showed a decrease in activation (by a reduction in NF-κB nuclear translocation) on stimulation with HMGB1 however it was shown that wild-type and TLR2 knockout mice responded similarly to HMGB1 suggesting that TLR4 is the more predominant receptor (Kokkola et al., 2005). Such findings indicate that TLR2 and TLR4 are the major receptors for HMGB1 but other receptors such as RAGE also play a contributory role (Park et al., 2004).

1.7.2 Receptor for advanced glycation end products (RAGE)

RAGE is a 45-55kDa multiligand member of the immunoglobulin superfamily of cell surface molecules capable of interacting with a broad spectrum of ligands such as advanced glycation endproducts (AGEs), S100B/calmodulin, amyloid β-peptides and HMGB1 (Schmidt et al., 2000; Bucciarelli et al., 2002; Stern et al., 2002). In normal adult tissues, RAGE is expressed at low levels, except in the lung where it is abundant; however, it becomes rapidly upregulated at sites where its ligands accumulate and can produce a sustained cellular activation through multiple intracellular signalling pathways (Raucci et al., 2008). Its involvement in inflammation has been suggested by many findings. First, RAGE is upregulated in all inflammatory lesions studied, including rheumatoid arthritis (Hofmann et al., 2002), inflammatory kidney disease (Abel et al., 1995), arteriosclerosis (Hofmann et al., 2001) and inflammatory bowel disease (Hofmann et al., 1999). As a receptor of various ligands, RAGE has been implicated as a receptor on macrophages and a variety of tumour cells that mediate the cytokine activity of HMGB1 and is expressed on endothelium, smooth muscle cells, monocytes/macrophages, neurons and tumour cells (Brett et al., 1993; Stern et

al., 2002; Fiuza et al., 2003; Yang et al., 2005). The part of HMGB1 responsible for interaction with RAGE has been defined as amino acids 150-183, just before the C-terminal acidic tail (Dumitriu et al., 2005). RAGE is composed of a number of distinct protein domains; an extracellular region composed of three immunoglobulin-like domains, including an Ig-like V-type domain, which contains the ligand binding site, and two Ig-like C2-type domains. After the extracellular domain there follows a single transmembrane-spanning domain and a short 43-amino acid cytoplasmic tail (Figure 1.6).

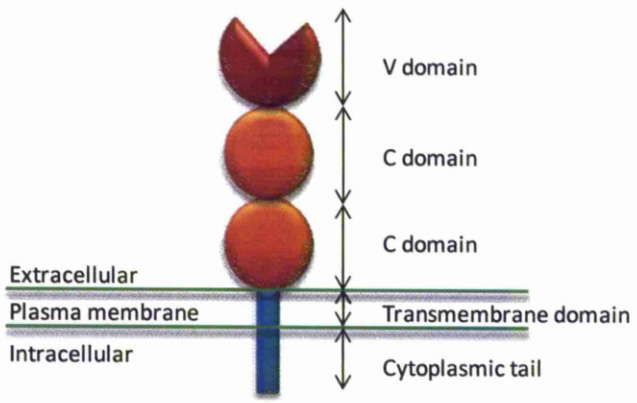

Figure 1.6: Schematic representation of the structural domains of RAGE.
RAGE contains an intra-cellular domain, a short transmembrane domain and an extracellular domain consisting of three immunoglobulin-like regions, one 'V' type followed by two 'C' types (adapted from Hudson et al., 2008).

It has been identified that RAGE has several isoforms deriving from alternative splicing. RAGE exists as a full-length isoform that is bound to the cell surface membrane, which contains the extra- and intracellular domains and soluble isoforms. One variant is a secreted form that lacks the transmembrane domain called soluble RAGE (sRAGE). It has been identified that the primary splicing mechanism of producing sRAGE is from the alternative splicing of intron 9/exon 10 of the RAGE gene (Yonekura et al., 2003). The other mechanism responsible for producing the circulating pool of sRAGE derives from membrane-bound full-length RAGE by proteolytic processing (Galichet et al., 2008; Raucci et al., 2008). sRAGE appears to function as a decoy receptor that neutralises the signalling of circulating ligands. Due to this, recombinant sRAGE has been used extensively and successfully in animal models for the treatment of inflammation-mediated diseases, including type II collagen-

induced arthritis (Hofmann et al., 2002) and diabetic atherosclerosis (Park et al., 1998; Goova et al., 2001).

Engagement of a ligand with RAGE results in the activation and transduction of multiple cell signalling mechanisms, such as the mitogen-activated protein (MAP) kinase family (p38, Erk 1/2 and jnk) and GTPases (Rac and CDC42) (Stern et al., 2002; Ishihara et al., 2003). One of the main signalling pathways is the activation of Rac and CDC42, which are guanosine triphosphatases that regulate cell motility and neurite outgrowth. The other pathway involves the subsequent activation and nuclear translocation of NF-κB via several MAPKs (Yang et al., 2005). Binding of HMGB1 to RAGE on murine macrophages and human neutrophils causes the phosphorylation of the p38 and p42/44 kinases, c-Jun N-terminal kinase and Erk1/2 (extracellular-signal-regulated kinase) which leads to activation of the NF-κB signalling pathway which promotes cell survival and cytokine production such as TNF-α, IL-6 and IFN-γ (Li et al., 2003; Park et al., 2003; Lotze et al., 2005; Yang et al., 2005; Ulloa et al., 2006). In endothelial cells, HMGB1-RAGE induces a transient phosphorylation of MAP kinases and expression of adhesion molecules resulting in amplification of the inflammatory response (Kokkola et al., 2005). Activation of these MAP kinases was also found to be RAGE independent, as no differences in signalling were observed in RAGE null mice (Kokkola et al., 2005; Ulloa et al., 2006). Despite there being no differences in signalling in RAGE null mice, HMGB1 induced secretion of pro-inflammatory cytokines was reduced by 70%. This suggests that although RAGE has been determined to be important for inflammatory responses, other receptors such as TLRs are involved in HMGB1 signalling (Ulloa et al., 2006). One unique feature of RAGE-induced NF-κB activation is the positive feedback loop in which the RAGE signal is maintained and amplified. Due to its ability to sustain cellular activation, RAGE can function as a master switch, converting short-lasting proinflammatory responses into long-lasting cellular dysfunction (Bierhaus et al., 2001). Targeting RAGE and blocking signalling could be a useful therapeutic treatment in dampening down the inflammatory response in drug-induced hepatotoxicity. Moreover, a recent study demonstrated that blockade of RAGE led to increased survival and a reduction in oxidative stress in APAP-treated animals (Ekong et al., 2006).

1.8 AIMS OF THESIS

The innate immune response has been shown to play an important role in the exacerbation of liver injury during APAP hepatotoxicity downstream of drug metabolism (Ju et al., 2002; Liu et al., 2004; Liu et al., 2006). However, despite intense research, inconsistencies and conflicting reports remain on the extent of the role of the innate immune and inflammatory response in the modulation of liver injury, with the key signalling pathways connecting dying hepatocytes and the inflammatory response needing further elucidation. The overall aim of this thesis was to assess the contribution of the inflammatory response in the progression of DILI by using appropriate animal models and utilising model hepatotoxins that can address and define the signalling pathways from activated immune and necrotic cells that lead to an inflammatory response. By this, we could test the hypothesis that the inflammatory response exacerbates APAP-induced liver injury via signalling between damaged hepatocytes and cells of the innate immune system.

The aims to answer the following questions and hypotheses of this thesis are:

- To define and characterise a suitable set of *in vivo* models of DILI and inflammation that could produce an inflammatory response which could be assessed and characterised by a chosen set of observable or measurable parameters.
    - What is the effect of overnight fasting on hepatotoxicity and the inflammatory response in a model of APAP-induced liver injury? Does fasting exacerbate these responses?

- To further evaluate the contribution of the innate immune response in the pathogenesis of APAP-induced liver injury by firstly identifying the role dimethyl sulfoxide (DMSO) may play in modulation of immune cell accumulation. Secondly, by using aspirin as a pharmacological inhibitor of the Nalp3 inflammasome in our models of hepatotoxicity and inflammation.
    - Does DMSO have an effect on APAP-induced hepatotoxicity and inflammation by promoting neutrophil recruitment?
    - Can aspirin help protect against DILI by inhibiting the associated inflammatory response?

- To further explore the signalling events triggered by DILI by investigating the effect of ethyl pyruvate (EP) as a pharmacological inhibitor of early (TNF-α, IL-6 and IL-1β) and late (HMGB1) inflammatory cytokines on the pathogenesis of APAP-induced liver injury.
    - Can EP protect against APAP hepatotoxicity by inhibition of the inflammatory response? Does EP prevent cytokine release in our models?
    - How does the effect of EP compare to the effect of complete inhibition of HMGB1 by the use of an anti-HMGB1 antibody?

- To evaluate the expression of the HMGB1 receptor RAGE in the tissues of APAP-treated mice and its relationship to the pathophysiology of APAP hepatotoxicity at the mRNA, protein and tissue level.
    - Is RAGE upregulated in response to danger signals such as HMGB1 that are released by necrotic cells during APAP-induced liver injury?

The studies in this thesis have been undertaken in an attempt to provide more information on the role of the innate immune and inflammatory response during DILI. The investigations are to assess the contribution of inflammation by targeting certain points in the signalling pathway between dying hepatocytes and cells of the innate immune system. The overall objective is to aid the development of effective methods of therapeutic intervention by understanding the relationship between inflammation and toxic outcome.

CHAPTER TWO

DEVELOPMENT OF AN INTEGRATED ANIMAL MODEL OF INFLAMMATION IN DRUG-INDUCED HEPATOTOXICITY

CONTENTS

2.1 INTRODUCTION

Currently, sufficient data to support an unequivocal role for inflammation in some cases of DILI are lacking, and in many cases, mechanistic details are unknown. Evidence to support the hypothesis that DILI triggers the activation of the innate immune system has been obtained from experimental *in vivo* models. The advantages of investigating inflammation in animal models of DILI are the stable genetic, molecular and immunological backgrounds of inbred strains. This lack of variation enables a more complete exploration and determination of possible inflammatory mechanisms contributing to the overall pathophysiology.

A widely studied model of DILI has been APAP-induced liver injury in mice. Evidence suggests that the initial hepatocellular damage caused by NAPQI may lead to the release of cellular contents known as Damage-Associated Molecular Patterns (DAMPs). These include HMGB1 and heat-shock proteins (HSP), which act as signals that activate cells of the innate immune system (Yu et al., 2006; Bianchi et al., 2007). This in turn leads to hepatic infiltration of inflammatory cells, which may contribute to the progression of liver injury by producing proinflammatory mediators such as cytokines and chemokines, as well as reactive oxygen and nitrogen species (Jaeschke et al., 2005; Ishida et al., 2006; Liu et al., 2006). Several different models of APAP-induced liver injury are being investigated, in which inflammation has been modulated in an attempt to explore the contribution of different processes of inflammation and their roles in DILI (Ju et al., 2002; Blazka et al., 1995; Liu et al., 2006). The variations between these models have however, lead to conflicting reports regarding the roles and contribution of particular immune and inflammatory mediators.

Many cell types have been implicated in determining the extent of liver injury, such as Kupffer cells (Ju et al., 2002), NK and NKT cells (Liu et al., 2004) and neutrophils (Liu et al., 2006). Neutrophils accumulating in areas of hepatocyte necrosis in the liver after APAP overdose were first recognised by Mitchell et al., 1973, but the relevance of this accumulation to the pathophysiology is still unclear (Cover et al., 2006). Increasing evidence has implicated neutrophils as the main type of leukocyte involved in the pathogenesis of APAP-induced liver injury (Jaeschke et al., 2002; Liu et al., 2006). A study showed that depletion of neutrophils using an anti-Gr-1 antibody significantly protected mice against APAP-induced

liver injury, as evidenced by markedly reduced serum ALT levels and centrilobular hepatic necrosis, and improved mouse survival (Liu et al., 2006). Another study demonstrated that the magnitude of liver injury induced by APAP correlated with hepatic neutrophil number and myeloperoxidase activity, suggesting that neutrophil recruitment was involved in the pathogenesis of APAP-induced liver injury (Ishida et al., 2002). These findings are in contrast to other studies with conflicting views concerning the role of neutrophils in APAP-induced liver injury (Lawson et al., 2000; Cover et al., 2006). One study suggested that although the numbers of neutrophils were increased in the liver from APAP-challenged animals, neutrophils did not contribute to the initiation and progression of APAP-induced liver injury. The inflammation observed after overdose was stated as not being severe enough to cause additional damage (Lawson et al., 2000).

The involvement of various proinflammatory mediators such as TNF-α (Ishida et al., 2004; Blazka et al., 1995), IL-6 (Bourdi et al., 2007) and IL-1β (Imaeda et al., 2009) in promoting tissue damage has been demonstrated. These and other cytokines are released into the bloodstream from the liver during hepatic toxic injury, with rapid changes in cytokine levels occurring within a few hours following DILI and may precede the formation of visible histological liver necrosis and release of serum ALTs (Lacour et al., 2005).

In addition to APAP, other model hepatotoxins have been used in animal models to investigate the role inflammation plays during DILI, such as ethanol (Adachi et al., 1994; Arteel et al., 1996; Yin et al., 1999; Wheeler et al., 2001), halothane (Bourdi et al., 2002b) and galactosamine (GalN) (Tiegs et al., 1990; Nowak et al., 2000). The bacterial endotoxin LPS, a potent inducer of inflammation, has been widely used in scientific investigations for many years as a tool to induce and study inflammatory responses (Roth et al., 1997). LPS administration induces systemic inflammation that mimics many clinical features of sepsis, including increases in the blood of proinflammatory cytokines such as TNF-α and IL-1 (Wichterman et al., 1980; Michie et al., 1988). The release of these mediators can lead to the activation and recruitment of innate immune cells in the liver, which propagate tissue damage. Neutralizing antibodies against TNF-α or IL-1 in LPS-treated animals has resulted in improved outcomes for this model (Tracey et al., 1987; McNamara et al., 1993). LPS has recently been used as an adjuvant to model hepatotoxins such as APAP, in order to test if

inflammation can increase sensitivity to DILI. In one study, low doses of APAP (100-400mg/kg, i.p) administered 2hr after a dose of LPS (44×10^6 EU/kg) to fasted C57BL/6 mice was shown to produce a leftward shift of the dose-response curve for APAP-induced liver injury (Maddox et al., 2010). The authors observed no hepatotoxicity with LPS alone although there was significantly greater TNF-α production. Histopathologic examination revealed centrilobular necrosis with marked neutrophilic accumulation only in livers of mice treated with LPS and APAP (Maddox et al., 2010). GalN has also been used as a model hepatotoxin (Izu et al., 2007) or as an accompaniment to LPS in a hepatotoxic and endotoxic shock model in mice (Galanos et al., 1979; Nowak et al., 2000). High doses of GalN induce fatal liver injury that histologically resembles human hepatic failure (Keppler et al., 1968) and induces an inflammatory response in the liver that resembles the reaction seen clinically in viral hepatitis (Medline et al., 1970). Moreover, when given concurrently with a low dose of LPS, GalN highly sensitizes animals to develop lethal liver injury resembling fulminant hepatitis (Galanos et al., 1979). GalN's mechanism of toxicity is different to that of APAP, causing hepatic necrosis by UTP depletion, leading to inhibition of RNA synthesis (Decker and Keppler, 1974). In addition, GalN induces oxidative stress, causes membrane damage, and can lead to a number of pathological disorders (El-Mofty et al., 1975; Han et al., 2006). The involvement of inflammation in the pathogenesis of GalN hepatotoxicity has been previously demonstrated, for example, one study showed that glycine protected against GalN toxicity as it inhibited the activation of Kupffer cells and subsequent cytokine release (Stachlewitz et al., 1999). Both LPS and GalN are useful tools to produce experimental inflammation in animals, with different mechanisms of cellular injury to those of APAP.

Given the complexity of interplay between chemically mediated toxicity and inflammation, and the differences observed between strains of mice, species and *in vivo* model pre-treatments, further investigation is required to define the precise role of cytokines and inflammatory cells in response to injury or as part of the inflammatory response.

The aims of the studies presented in this chapter were firstly to define a set of different *in vivo* models of DILI which could produce a comparable inflammatory response as determined by a chosen set of inflammatory parameters. Inflammation was determined in our models by neutrophil accumulation in the liver and by measurement of serum levels of the

cytokines TNF-α, IL-6 and IL-1β. The type of immune activation that APAP causes was characterised, and the model inflammatory stimulator LPS and hepatotoxin GalN were investigated as tools with which to compare the mechanism of toxicity and inflammation to that of APAP. Both have been shown to trigger an inflammatory response in mice, but without requiring metabolic activation into a toxic reactive metabolite, which is the main mechanism of APAP toxicity. These models would give mechanistic data to understand the nature of the immune response to APAP. The parameters of toxicity (serum ALT, histology), metabolism (hepatic GSH content) and inflammation (neutrophil accumulation, serum TNF-α, IL-6 and IL-1β) were defined for each model. The effect of increasing the dose of APAP on inflammatory cell accumulation was also investigated; testing the hypothesis that increased toxicity would drive APAP-induced necrosis and promote a more vigorous inflammatory response. The final aim of this chapter was to assess the effect fasting had on the APAP model of toxicity and inflammation after recent evidence suggested that fasting could inhibit APAP-induced apoptosis by ATP depletion, thereby promoting an inflammatory response (Antoine et al., 2010). The purpose of developing and defining these models was to aid the investigation of particular pathways of inflammation downstream from initial cellular injury and evaluate if modulation of these pathways could reduce the level of overall injury.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Infinity ALT liquid kits were purchased from ALPHA Laboratories (Eastleigh, U.K). Unless otherwise stated, all other chemicals and materials were purchased from Sigma-Aldrich (Poole, U.K).

2.2.2 Experimental animals

The protocols described were in accordance with criteria outlined in a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Animals were purchased from Charles River laboratories and had a 5 day acclimatisation period prior to experimentation. Animals were also maintained in a 12hr light/dark cycle with free access to food and water.

2.2.3 Animal dosing regime

Male C57BL/6 mice (18-23g) were either fasted overnight with free access to water or had free access to food and water prior to experimentation. Mice were administered a single *i.p.* injection of APAP (0-1000mg/kg) in 0.9% saline then were euthanized after 5hr. Other groups received APAP (530mg/kg) in 0.9% saline and were euthanized between 0-24hrs. Some mice received LPS (0-5mg/kg *i.p.*) in 0.9% saline or GalN (0-1500mg/kg *i.p.*) in 0.9% saline and were then euthanized between 0-24hr post dose. Control animals received 0.9% saline. Treatment groups consisted of 4-5 individual animals.

2.2.4 Assessment of hepatotoxicity in treated male C57BL/6 mice

Animals were sacrificed by CO_2 inhalation and confirmed by cervical dislocation. Blood was collected via cardiac puncture and allowed to clot overnight at 4°C. Livers were removed, rinsed in ice cold 0.9% saline and a section was fixed in 10% neutral buffered formalin. The

rest of the liver was snap frozen in liquid nitrogen. Serum was separated by pulse centrifugation from blood and alanine transaminase (ALT) levels were determined.

2.2.5 Determination of hepatic glutathione levels in C57BL/6 mouse whole liver during drug-induced hepatotoxicity

Liver samples were prepared by homogenisation of 30-50 mg of tissue from the major lobe in 5-sulfosalicyclic acid (200µl; 6.5 % w/v) and GSH stock buffer (800µl; 143mM NaH_2PO_4, 6.3mM EDTA, pH 7.4) and the protein was allowed to precipitate on ice for 10 min prior to centrifugation (14000rpm for 5 min). Liver protein samples were dissolved in 1ml 1M NaOH prior to protein content determination. Supernatants containing GSH and samples for protein determination were stored at -80°C until analysis. Total GSH content was measured spectromically (412nm) by a kinetic reaction that took place between total GSH and GSH reductase on a Dynatech Laboratories UV-visible absorbance MRX microtiter-plate reader (Vandeputte et al., 1994). The results were compared to GSH standards (0-80nm/ml). Levels of GSH were normalised to hepatic protein content using Bio-Rad protein assay reagent according to the manufacturer's instructions.

2.2.6 Preparation of histology sections

Histological specimens were prepared in the Histology Laboratory, Veterinary Laboratory Services, School of Veterinary Science, University of Liverpool. Slices (3-4mm) of the major lobe of the liver were immersed in neutral buffered formalin (10%, Sigma) for 24hr. Transverse sections were taken from the formalin fixed sample, and placed cut side down into a pre-labelled histology cassette. Tissue samples were processed using a tissue processor (Tissue-TEK Vacuum Infiltration Processor). The tissue processor removes water from the tissues and replaces it with paraffin wax which solidifies tissue and enables it to be cut. Briefly, the tissue was dehydrated with a series of increasing concentrations of ethanol to remove free and bound water. They were then submersed and washed in xylene which removes the ethanol. The tissue specimens were then removed from their cassette and embedded into a mould of molten paraffin wax with the trimmed side down. The cassette was then placed on top of the mould and topped up with wax and allowed to solidify. 3-5µm thick

sections were cut and floated onto a clean water bath (45°C) to remove creases. The sections were retrieved with a charged microscope slide and allowed to dry in an oven.

2.2.7 Histological and immunohistological staining of liver sections

For histological assessment, sections were stained with haematoxylin and eosin (HE). For the assessment of hepatocellular glycogen contents, sections from selected livers were stained with the Periodic Acid Schiff (PAS) reaction. On selected cases, immunohistology for the confirmation of apoptosis was performed by staining for cleaved caspase-3 as previously described (Antoine et al., 2009). All stains were performed by technical staff in the Histology Laboratories, Veterinary Laboratory Services, School of Veterinary Science, University of Liverpool.

2.2.8 Histological examination of C57BL/6 mouse liver

The histological examination was undertaken in collaboration with Prof A Kipar, Veterinary Pathology, School of Veterinary Science, University of Liverpool. HE-stained sections were examined and the degree of hepatocyte loss and necrosis was scored according to the criteria in table 2.1. Leukocyte infiltration was assessed and recorded. PAS-stained sections were used to assess the hepatocellular glycogen content, and sections stained for cleaved caspase-3 served to confirm hepatocyte death via apoptosis. The histological assessment was undertaken blindly by Prof A Kipar.

Table 2.1: Scoring of histological changes in murine livers (based on Antoine et al., 2009)

Score	Definition
0	Normal – no evidence of hepatocyte necrosis
1	Hepatocyte loss and necrosis – minimal to mild Focal Limited to centrilobular region Less than ¼ of affected lobules are necrotic Associated with vacuolar degeneration or haemorrhages
2	Hepatocyte loss and necrosis – mild to moderate Focal or multifocal Extends from central to midzonal lobular region ½ of affected lobules are necrotic Associated with vacuolar degeneration or haemorrhages
3	Hepatocyte loss and necrosis – moderate to severe Multifocal May extend from centrilobular region to portal area More than ½ to ¾ affected lobules are necrotic Associated with vacuolar degeneration or haemorrhages
4	Hepatocyte loss and necrosis – severe Multifocal More than ¾ affected lobules are necrotic Associated with vacuolar degeneration or haemorrhages
5	Massive Hepatocyte loss and necrosis – severe, involving entire lobules Hepatocyte loss extends from central vein to portal area Hepatocyte loss extends to adjacent lobules (multilobular necrosis) Associated with vacuolar degeneration or haemorrhages

2.2.9 Measurement of cytokines in serum from animals dosed with LPS, GalN and APAP using Luminex Analysis

Serum was collected from control and treated animals. Concentrations of TNF-α, IL-6 and IL-1β were measured using the Bio-Plex Assay kit (Bio-Rad Laboratories, Inc).

A filter plate was wet with 100µl of Assay Buffer per well, then a vacuum manifold was applied to the bottom of the plate. The bottom of the plate was then dried thoroughly by blotting on a clean paper towel. The coupled magnetic beads were vortexed at medium speed for 30sec then 50µl were added to each well. The plate was washed twice with 100µl of Wash Buffer and the vacuum manifold. The standards and samples were gently vortexed for 1-3 sec before 50µl were added to the appropriate well. The plate was covered with a sheet of sealing tape to prevent liquid escaping from the wells. The plate was then placed on a plate

shaker and covered in aluminium foil to protect from light. The plate was shaken at room temperature at 1,100rpm for 30sec, and then at 300rpm for 30min. During incubation, 1x detection antibodies were prepared by dilution of 10x detection antibodies with Detection Antibody Diluent. After incubation was complete, the sealing tape was removed and discarded. 100µl of Wash Buffer was added to each well and removed by the vacuum manifold. This last wash step was repeated 3 times, blotting excess liquid in between on a clean paper towel. The 1x detection antibodies were gently vortexed for 1-3sec before 25µl were added to each well. The plate was covered and vortexed briefly (1,100rpm for 30sec) before being incubated on the shaker at room temperature (300rpm for 30min). During incubation, 1x streptavidin-phycoerythin (streptavidin-PE) was prepared by dilution using Assay Buffer. After incubation, the sealing tape was carefully removed and discarded. The plate was washed 3 times with 100µl Wash Buffer using the vacuum manifold. The streptavidin-PE (1x) was vortexed vigorously for 3-5sec before 50µl was added to each well. The plate was once again covered and incubated on the plate shaker at room temperature, this time for 10min. After the streptavidin-PE incubation, the sealing tape was carefully removed and discarded. The plate was washed 3 times with 100µl Wash Buffer using the vacuum manifold. 125µl of Assay Buffer was added to each well. The plate was covered with a new sheet of sealing tape. The plate was shaken at room temperature at 1,100rpm for 30sec. The sealing tape was then removed before reading the plate on a Luminex 100 system. The results were acquired using Bio-Plex Manager software, a standard curve and 5-parameter logistics was used to analyse the data. The lower range of the assay is <5.8 pg/ml for TNF-α, <0.74pg/ml for IL-6 and <10.36pg/ml for IL-1β. Levels below these values were assigned a value of zero.

2.2.10 Statistical analysis

All results are expressed as mean ± standard deviation (S.D). Values to be compared were analysed for non-normality using a Shapiro-Wilk test. The unpaired t-test was used when non-normality was indicated. A Mann-Whitney U test was used for non-parametric data. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a *post* hoc Bonferroni test. All calculations were performed using SPSS statistical software, results were considered significant when $p<0.05$.

2.3 RESULTS

2.3.1 LPS model of hepatic inflammation in C57BL/6 mice

The dose and time dependent nature of LPS-induced effects on the liver and the associated inflammation was investigated in male C57BL/6 mice to assess the usefulness of LPS as a tool to induce inflammation and compare to other model hepatotoxins. In male C57BL/6 mice, there was no statistically significant change in serum ALT activity compared to control animals (41.37±8.45U/l) after administration of LPS over a dose range (0-5mg/kg) at 24hr post dose (Figure 2.1A). There was also no significant effect on hepatic GSH content induced by LPS at 24hr post dose compared to control levels (73.68±9.53nmol/mg) (Figure 2.1B). No significant effect on serum ALT activity was observed with LPS over the time course investigated (0-24hr) (Figure 2.1C) or on hepatic GSH content (Figure 2.1D). With a dose level of 5mg/kg, there was a significant increase in the serum level of TNF-α at 24hr (513.13±50.15pg/ml) (Figure 2.2A). IL-6 also increased at 24hr after LPS (1201.12±157.20pg/ml) (Figure 2.2B) as well as levels of IL-1β (227.08±36.93) (Figure 2.2C). The histological evaluation undertaken on the livers of animals dosed with LPS (5mg/kg) and euthanized after 0 to 24hr revealed no evidence of hepatocyte necrosis (score 0, see Table 2.1) but identified neutrophil influx of variable degree from 3hr post dosing on. There was also some evidence of potential hepatocyte damage represented by the loss of glycogen from 3hr post dosing onwards.

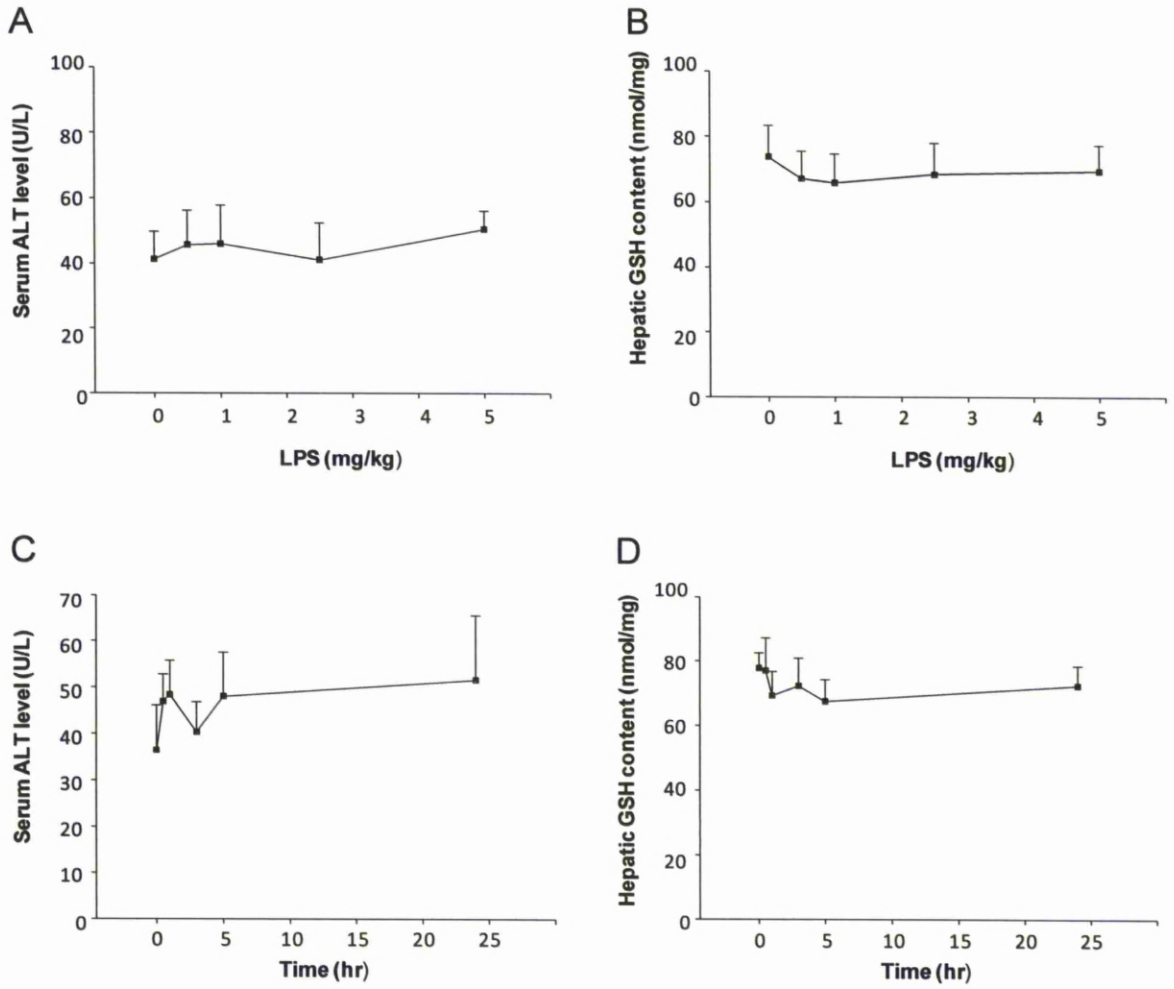

Figure 2.1: LPS 24hr dose response and time course.
The dose effect of LPS (0-5mg/kg) on serum ALT activity (A) and hepatic GSH content (B) was assessed at 24hr. The time dependent effects of LPS (5mg/kg) on serum ALT activity (C) and hepatic GSH content (D) were assessed over 0-24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to saline treated controls.

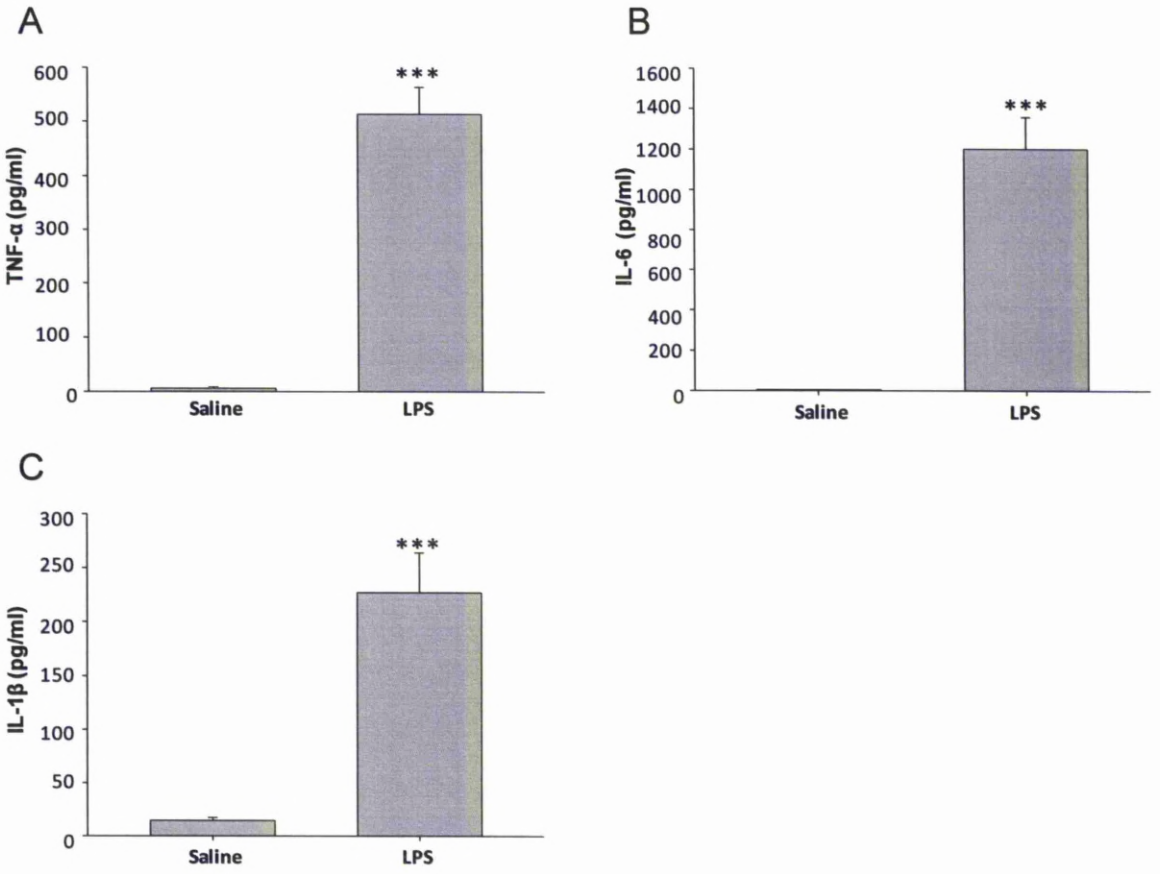

Figure 2.2: Serum cytokine levels in male C57BL/6 mice after LPS exposure.
Male C57BL/6 mice were administered LPS (5mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to saline treated controls. ***$p < 0.001$.

Table 2.2: Histological findings after LPS treatment. The time dependent effects of LPS (5mg/kg) were assessed over 0-24hr, using the scoring scheme of table 2.1. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 4 animals per group.

Time post dosing	Score	Histological findings
0hr	0	Diffuse glycogen in hepatocytes; no histological abnormality is recognised (NHAIR)
0.5hr	0	Diffuse glycogen in hepatocytes; NHAIR
1hr	0	Diffuse glycogen; NHAIR
3hr	0	Reduced hepatocellular glycogen; increased numbers of neutrophils (NL) between hepatic cords; several central veins with some NL in lumen; scattered small NL aggregates between hepatic cords; focal mixed cellular infiltration, relatively large, extending focally from one central vein
5hr	0	Markedly reduced or no hepatocellular glycogen; several leukocytes in central veins and a few around them; scattered small random NL aggregates and generally increased leukocytes between hepatic cords .
24hr	0	No glycogen; several leukocytes in central veins and a few around them (Figure 2.3); scattered small random NL aggregates and generally increased leukocytes between hepatic cords

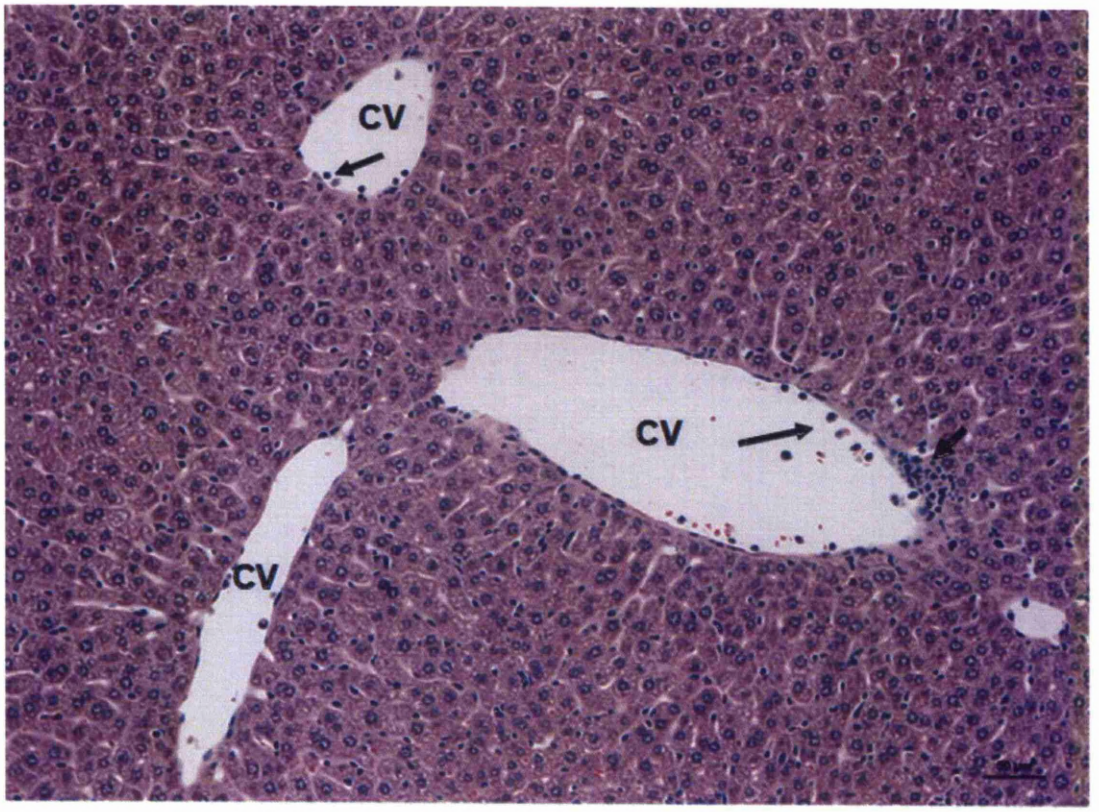

Figure 2.3: Histological findings in a C57BL/6 mouse at 24hr post LPS (5mg/kg) treatment.

Several neutrophils are present within the lumen and attached to endothelial cells of central veins (CV) (long arrows) and in the immediately adjacent parenchyma (short arrow). HE stain.

2.3.2 GalN model of hepatotoxicity and inflammation in C57BL/6 mice

The dose dependent effects of GalN were investigated in C57BL/6 mice at 5hr post treatment. GalN induced a dose dependent increase in serum ALT activity which was significant from control levels at 600mg/kg (83.16±15.43U/l) and 800mg/kg (197.78±26.08U/l) (Figure 2.4A). No significant change in hepatic GSH levels was observed compared to control levels over the dose range (Figure 2.4B). In order to determine the threshold of toxicity for GalN, the dose range was increased above 800mg/kg (0-1500mg/kg) and the time point for assessment extended to 24hr. A time course of the effect of GalN at 800mg/kg (0-24hr) was also performed. As before, GalN induced a dose dependent increase in serum ALT activity that was significant from control levels above 400mg/kg (Figure 2.5A). Increasing the time point to 24hr resulted in a greater level of serum ALT activity being observed at 600mg/kg (542.76±163.95U/l) and 800mg/kg (825.34±127.03U/l) compared to those corresponding doses at 5hr (83.16±15.43U/l and 197.78±26.08U/l, respectively). No significant change in the level of hepatic GSH content was observed at 24hr across the dose range (Figure 2.5B). GalN induced a time dependent increase in toxicity (serum ALT activity) with a dose of 800mg/kg. Serum ALT activity was significantly increased from control levels at 5hr (339.08±51.84U/l) and 24hr (875.30±144.81U/l) post GalN dose (Figure 2.5C). No significant change in hepatic GSH content was observed across the time course (Figure 2.5D). With a dose level of 800mg/kg, there was a significant increase in the serum level of TNF-α (56.44±5.68pg/ml) (Figure 2.6A), IL-6 (63.22±8.41pg/ml) (Figure 2.6B) and IL-1β (40.72±3.64) at 24hr (Figure 2.6C). The histological examination did not reveal any histological changes at low doses 24hr post treatment. With higher doses, random small areas of mixed cellular infiltrates with associated individual hepatocyte apoptosis and necrosis was observed (Figure 2.7; 24hrs 1500mg/kg; Table 2.3). The effect of a GalN dose that induced serum cytokine levels (800mg/kg) was studied over a period of 0-24hr post dosing. Up to 3hr post treatment, no histological abnormalities were detected. At 5hr, all animals showed evidence of slight hepatocellular dissociation and exhibited scattered apoptotic hepatocytes (Figure 2.8). A slight increase of neutrophils was seen between hepatic cords. At 24hr, multifocal random areas of hepatocyte necrosis, apoptosis, neutrophil and macrophage infiltration were seen (Table 2.4).

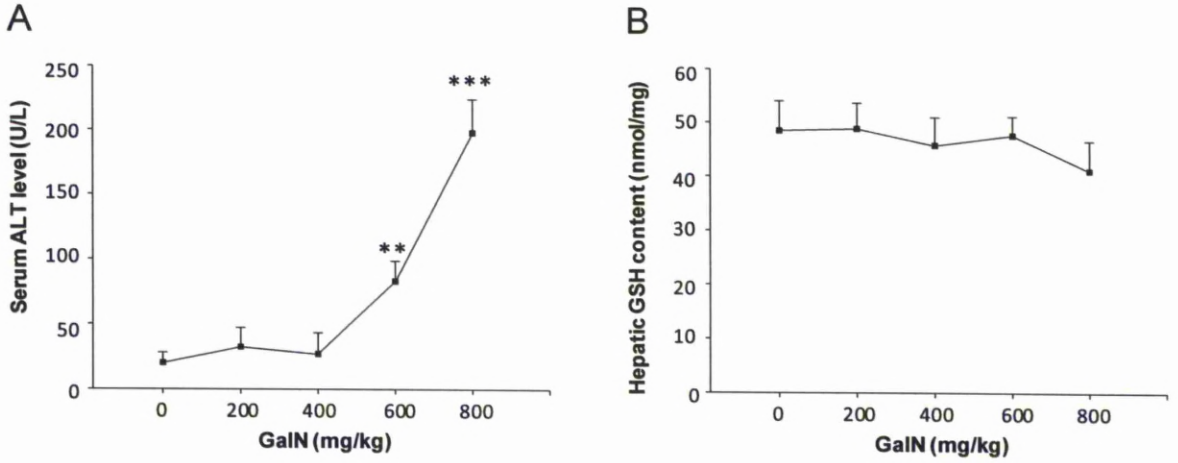

Figure 2.4: GalN-induced hepatotoxicity.
The dose effect of GalN (0-800mg/kg) on serum ALT activity (A) and hepatic GSH content (B) was assessed at 5hr in male C57BL/6 mice. Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to saline treated controls. $^{**}p < 0.01$ and $^{***}p < 0.001$.

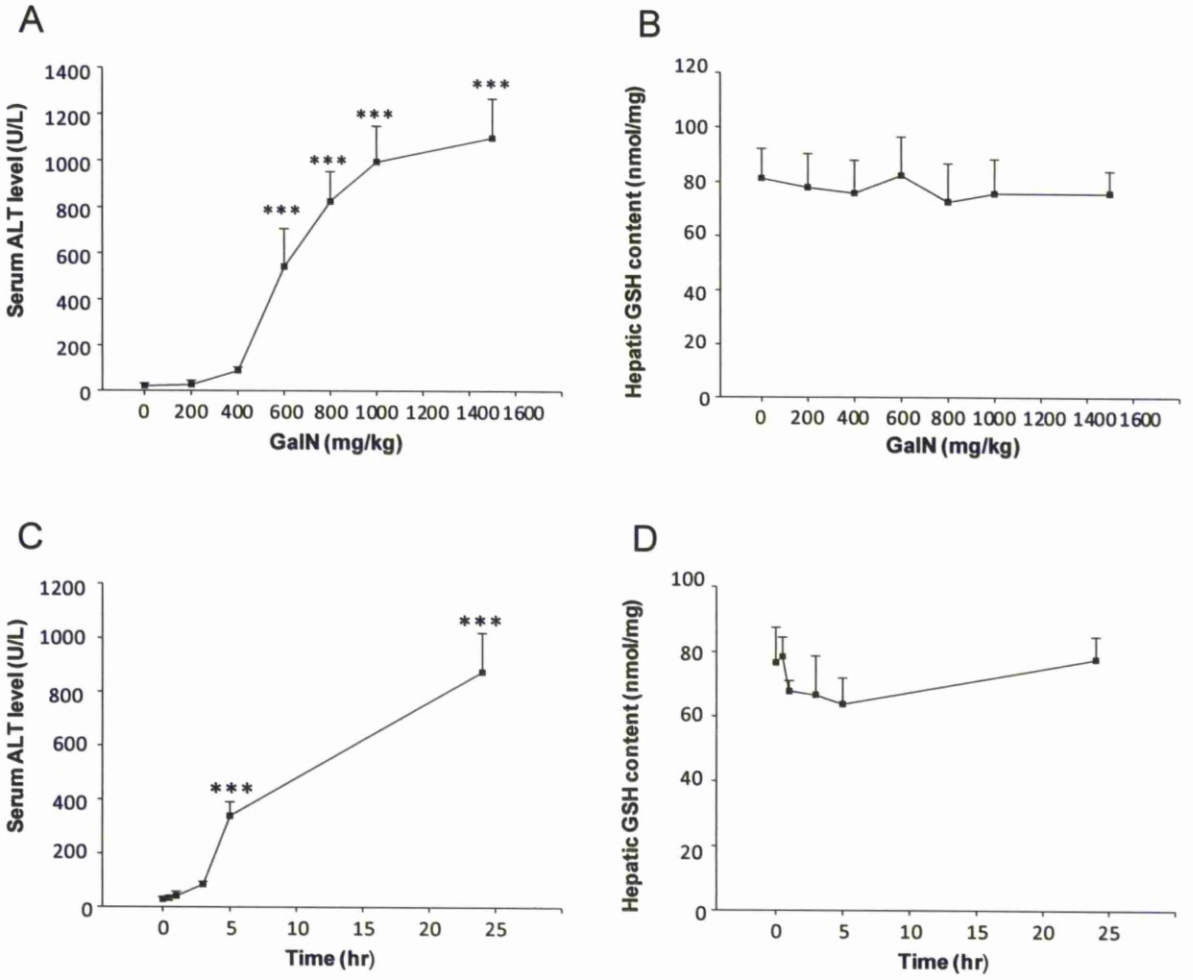

Figure 2.5: GalN 24hr dose response and time course.
The dose effect of GalN (0-1500mg/kg) on serum ALT activity (A) and hepatic GSH content (B) was assessed at 24hr in C57BL/6 mice. The time dependent effects of GalN (800mg/kg) on serum ALT activity (C) and hepatic GSH content (D) were assessed over 0-24hr. Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to saline treated controls. ***$p < 0.001$.

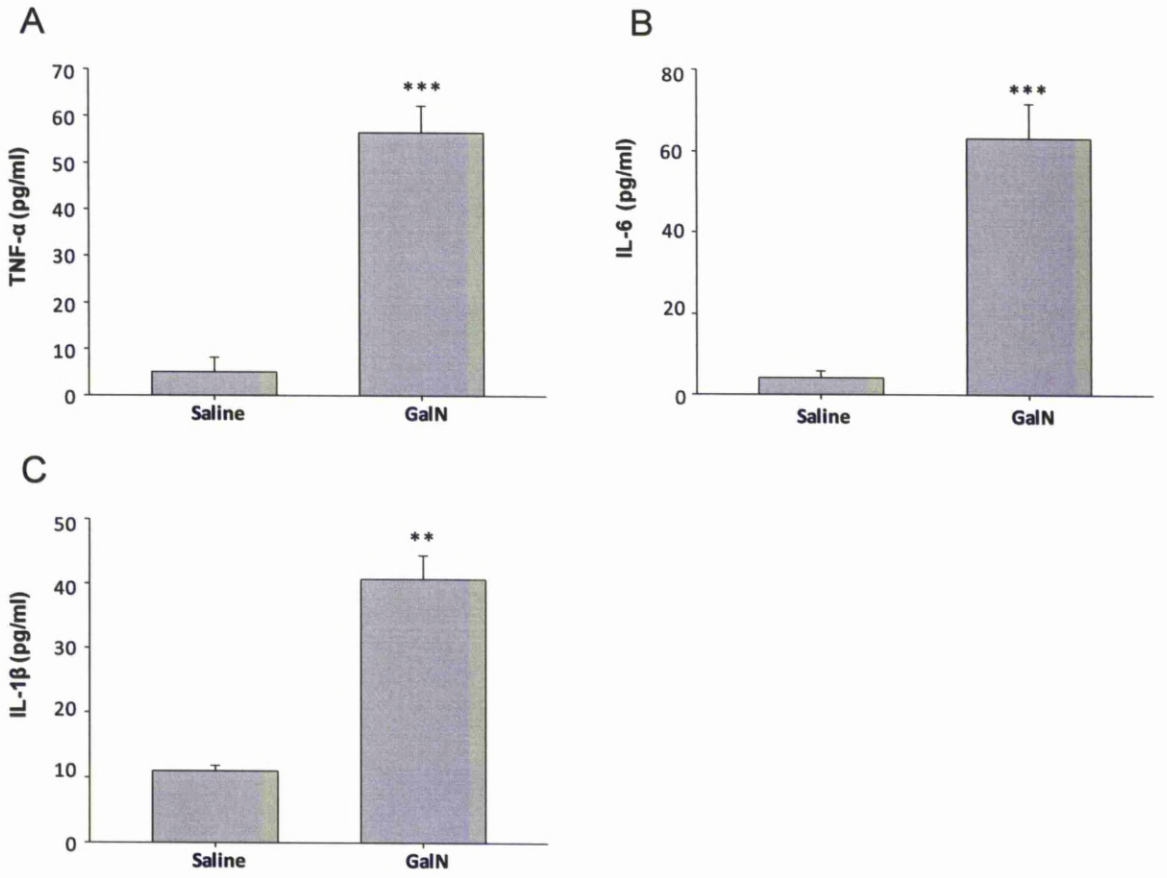

Figure 2.6: Serum cytokine levels during GalN-induced hepatotoxicity in male C57BL/6 mice.

Male C57BL/6 mice were administered GalN (800mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to saline treated controls. $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 2.3: Histological findings after GalN treatment. The dose effect of GalN (0-1500mg/kg) on male C57BL/6 mouse livers was assessed at 24hr. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 5 animals per group.

GalN Dose	Score	Histological findings
0mg/kg	0	Diffuse glycogen in hepatocytes; no histological abnormality is recognised (NHAIR)
200mg/kg	0	Diffuse glycogen in hepatocytes; scattered small mixed cellular aggregates with occasional necrotic hepatocytes (as in control animals)
400mg/kg	0	Diffuse glycogen in hepatocytes; scattered small mixed cellular aggregates with occasional necrotic hepatocytes (as in control animals)
600mg/kg	0	Diffuse glycogen in hepatocytes; disseminated small mixed leukocyte aggregates between hepatic cords (more intense than in controls and at lower doses)
800mg/kg	0	Diffuse glycogen; disseminated small mixed cellular aggregates with occasional necrotic hepatocyte; some random areas of coagulative necrosis with haemorrhage and neutrophil infiltrate, surrounded by rim of hepatocytes with loss of glycogen
1000mg/kg	0	Diffuse glycogen in hepatocytes; disseminated small mixed cellular aggregates with occasional necrotic hepatocyte; some random areas of coagulative necrosis with haemorrhage and neutrophil infiltrate, surrounded by rim of hepatocytes with loss of glycogen
1500mg/kg	0	Multifocal random areas with mixed leukocytes (neutrophils, macrophages, lymphocytes) with occasional apoptotic and necrotic hepatocytes (Figure 2.7A); some disseminated individual necrotic and apoptotic hepatocytes (Figure 2.7B)

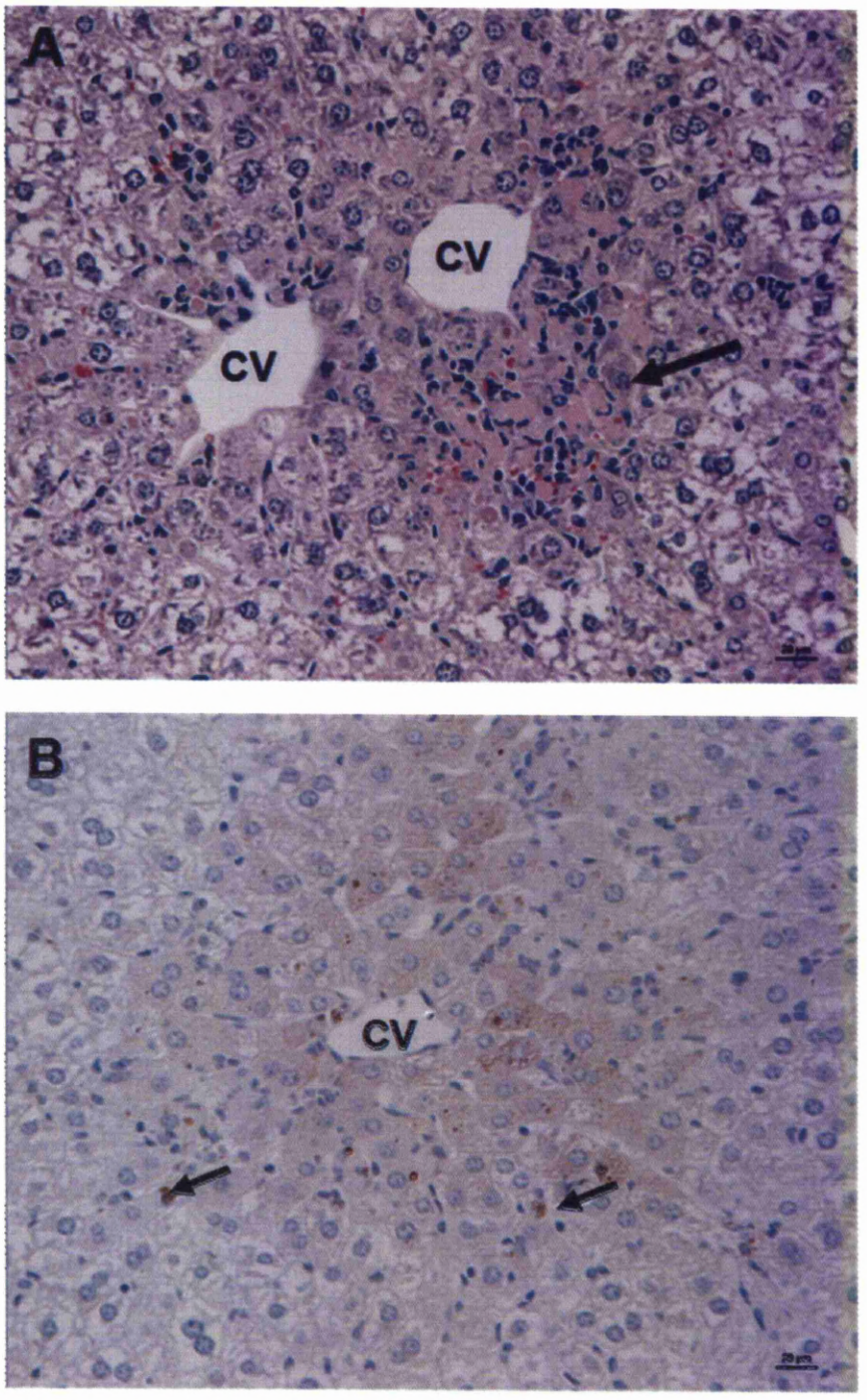

Figure 2.7: Histological findings in a C57BL/6 mouse at 24hr post GalN treatment (1500mg/kg).

(A) Focal area of leukocyte infiltration with some necrotic/apoptotic hepatocytes (arrow). HE stain. (B) Staining for cleaved caspase-3 identifies scattered apoptotic hepatocytes (arrows). Peroxidase anti-peroxidase method. Pananicolaou's haematoxylin counterstain.

Table 2.4: Histological findings at different time points after GalN treatment. The time dependent effects of GalN (800mg/kg) were assessed over 0-24hr post dose. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 4 animals per group.

Time post dosing	Score	Histological findings
0hr	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
0.5hr	0	Diffuse glycogen; (NHAIR)
1hr	0	Diffuse glycogen; (NHAIR)
3hr	0	Diffuse glycogen; (NHAIR)
5hr	0	Diffuse glycogen; slight hepatocellular dissociation; scattered apoptotic cells and bizarre mitotic figures in hepatocytes (Figure 2.8); occasional neutrophils between hepatic cords
24hr	0	Diffuse glycogen, but with patchy glycogen loss in association with multifocal random, variable sized (rel. large) focal areas of neutrophil and macrophage infiltration and hepatocyte necrosis/apoptosis; some leukocytes in central veins

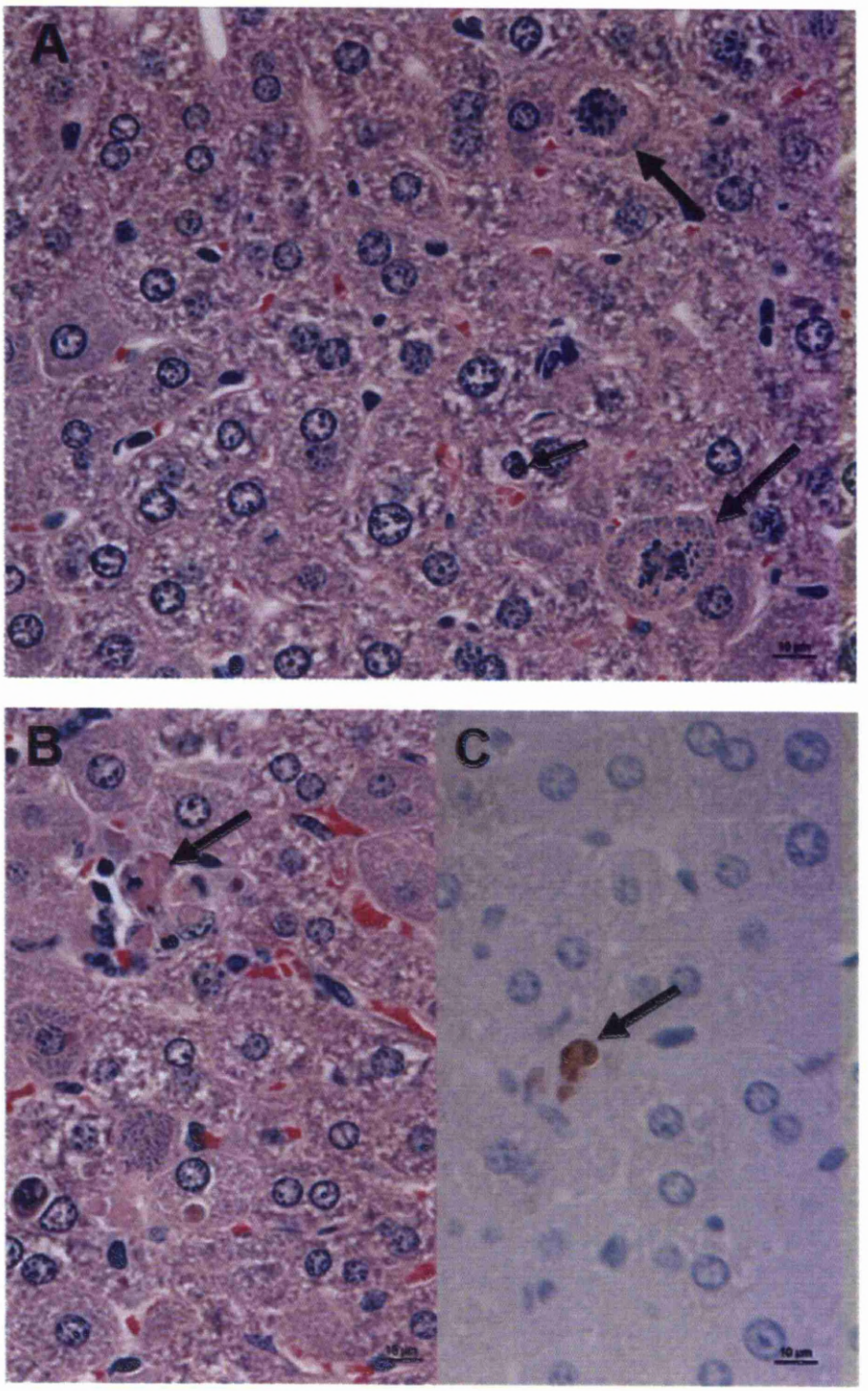

Figure 2.8: Histological findings in a C57BL/6 mouse at 5hr post GalN treatment (800mg/kg).

(A) Scattered apoptotic hepatocytes (small arrow) and hepatocytes with bizarre mitotic figures (large arrows) are seen. HE stain. (B) Closer view of apoptotic hepatocytes (arrow). HE stain. (C) Staining for cleaved caspase-3 confirms that dying hepatocytes undergo apoptosis (arrow). Peroxidase anti-peroxidase method. Pananicolaou's haematoxylin counterstain.

2.3.3 Effect of increasing APAP dose on hepatotoxicity and neutrophil recruitment in male C57BL/6 mice

APAP induced a dose dependent increase in serum ALT activity at 24hr which was significant from control levels at 530mg/kg (2126.09±263.42U/l) (Figure 2.9A). There was also a significant increase in toxicity at 530mg/kg from 300mg/kg. There was no significant decrease in hepatic GSH content between control and dosed groups at 24hr with GSH levels returned to control levels (Figure 2.9B). At 24hr there were no surviving mice subjected to a 750mg/kg dose of APAP, therefore ALT and GSH values were not included. The histological examination identified the typical APAP-induced centrilobular hepatocyte loss in all animals at a dose of 530mg/kg and 750mg/kg. After 300mg/kg, mild typical lesions were apparent in one mouse. In mice treated with 530mg/kg affected areas generally showed mild to moderate neutrophil infiltration at this stage (Figure 2.10). Remaining hepatocytes in affected areas exhibited marked hydropic degeneration and coagulative necrosis (Figure 2.10) and there were occasional apoptotic cells (Table 2.5).

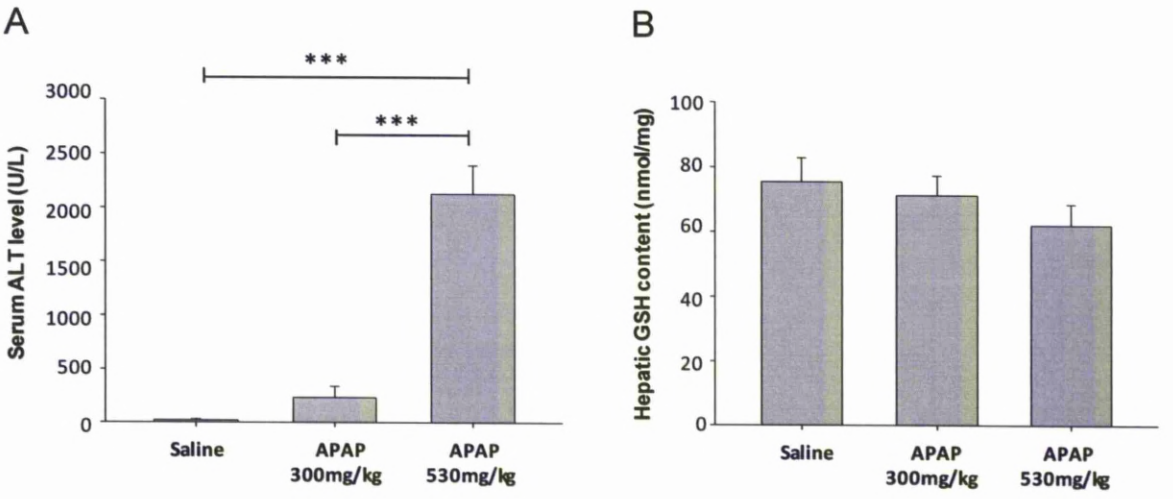

Figure 2.9: APAP-induced hepatotoxicity in male C57BL/6 mice.
The dose effect of APAP (0, 300, and 530mg/kg) on serum ALT activity (A) and hepatic GSH content (B) was assessed at 24hr. Data is given as mean ±S.D of 5 mice per group. Statistical significance was assigned relative to saline treated controls. ***$p < 0.001$.

Table 2.5: Histological findings in mice after APAP treatment (dose response). The dose effect of APAP (0-750mg/kg) on male C57BL/6 mouse livers was assessed at 24hr post dose. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed and the range of scores and average score in each of 5 or 6 (750mg/kg) animals per group.

APAP dose	**Score [average]**	**Histological findings at 24hr after dosing**
0mg/kg	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
300mg/kg	0(-1) [0.1]	Diffuse glycogen; NHAIR (4/5); one animal with centrilobular glycogen loss, hydropic degeneration and necrosis/apoptosis of hepatocytes and moderate neutrophil infiltration in affected areas
530mg/kg	1-4 [2.7]	Extensive centrilobular cell loss, hydropic degeneration, necrosis and apoptosis of remaining hepatocytes, with mild to moderate neutrophil infiltration in affected areas (Figure 2.10)
750mg/kg*	2-3 [2.83]	Centrilobular hepatocyte loss with scattered necrotic cells; no inflammatory infiltration

* The animals died maximally 10hr after dosing

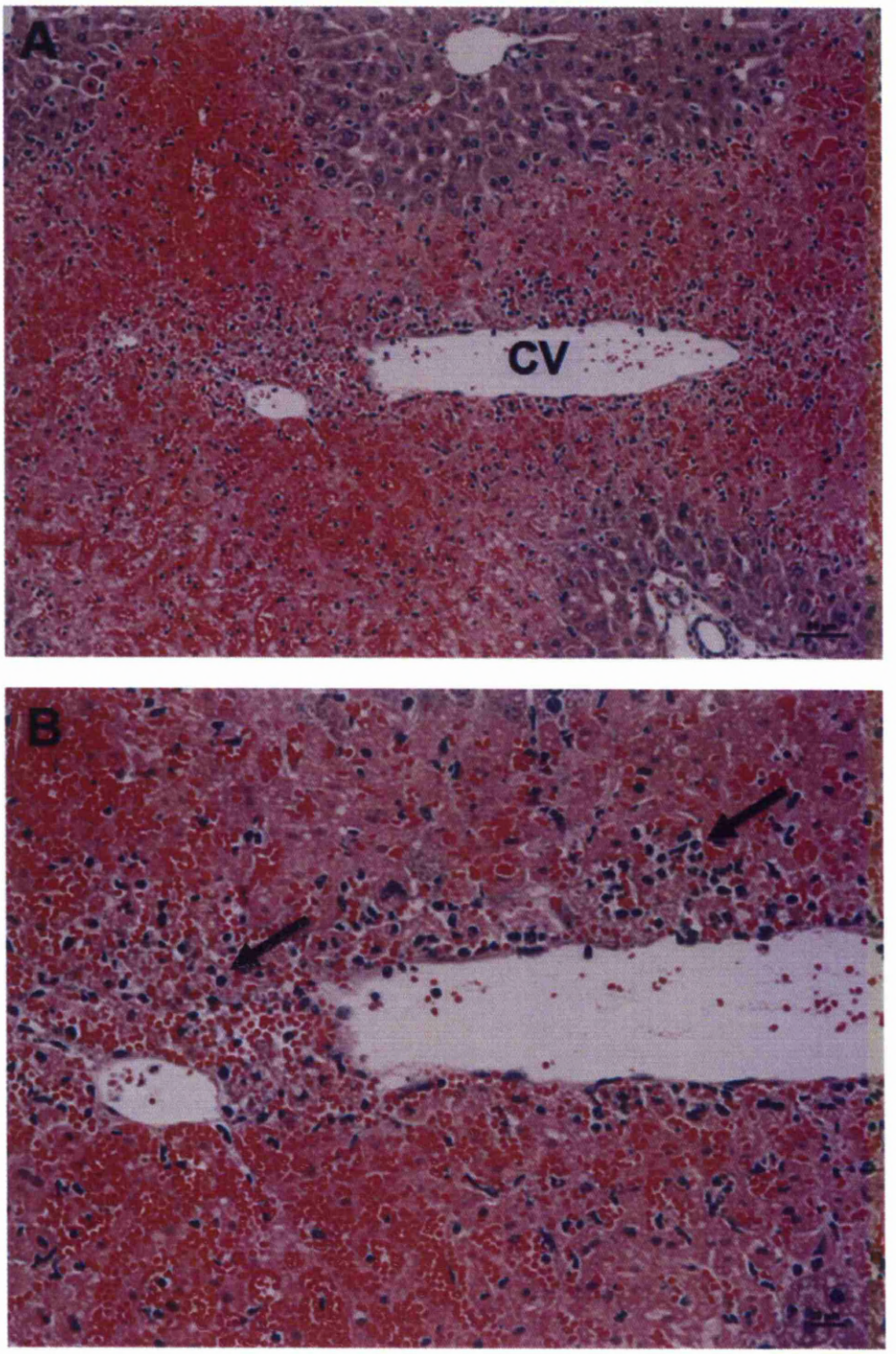

Figure 2.10: Histological findings in a C57BL/6 mouse at 24hr post APAP treatment (530mg/kg).

(A) There is extensive centrilobular cell loss with hydropic degeneration of remaining cells in affected areas, central vein (CV). (B) Closer view of (A), highlighting infiltration of neutrophils in affected areas (arrows). HE stains.

2.3.4 Effect of increasing APAP dose on survival of male C57BL/6 mice

Male C57BL/6 mice were administered either a 300mg/kg, 530mg/kg or 750mg/kg dose of APAP then euthanized at 24hr post dose. The 750mg/kg dose of APAP resulted in a significant increase in mortality starting at the 8hr mark (~66% survival) with 0% survival at 11hr (Figure 2.11). Mice subjected to a dose of 530mg/kg APAP displayed 100% survival until 19hr (~91%) and had ~83% survival by 24hr. The lower dose of APAP (300mg/kg) and control mice each had 100% survival at 24hr.

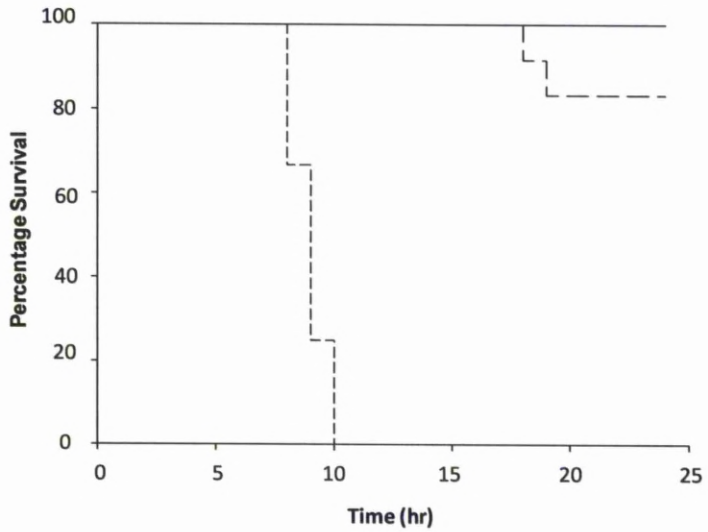

Figure 2.11: Kaplan-Meier Survival Curve.
Male C57BL/6 mice treated with APAP (0, 300, 530 and 750mg/kg) and percent (%) survival was recorded over 24hr (Saline, solid line ——; APAP 300mg/kg, dotted line ····; APAP 530mg/kg, large dashed line — —; APAP 750mg/kg, small dashed line - - -).

2.3.5 APAP model of hepatotoxicity and inflammation in C57BL/6 mice

In male C57BL/6 mice a dose dependent increase in serum ALT activity was observed at dose levels of 530mg/kg and above at 5hr (Figure 2.12A). APAP induced a dose dependent decrease in total hepatic GSH content which was significant from control levels at doses of 150mg/kg and above at 5hr (Figure 2.12B). The time dependent effects of APAP-induced hepatotoxicity, metabolism and cytokine release were also determined. Serum ALT activity was significantly increased from control levels at 3hr (1004.74±252.56U/l), 5hr (2056.82±214.47U/l) and 24hr (2702.49±231.65U/l) post APAP (530mg/kg) dose (Figure 2.12C). A significant decrease in hepatic GSH content was observed 30min post APAP dose (31.94±3.76nmol/mg) compared to vehicle control mice (73.57±2.88nmol/mg) (Figure 2.12D). Hepatic GSH content decreased further at 1hr (25.42±7.28nmol/mg) and reached 2% of control at 3hr (1.68±0.85nmol/mg) post APAP dose. Hepatic GSH content increased at 5hr post dose (17.78±8.77nmol/ml) and returned to control levels at 24hr (69.75±7.86nmol/ml). Serum TNF-α was significantly increased from control levels at 3hr (67.52±9.07pg/ml), 5hr (74.37±7.13pg/ml) and 24hr (75.86±7.94pg/ml) post APAP dose (Figure 2.13A). Serum IL-6 levels were significantly increased at 1hr (40.49±14.21pg/ml), reaching their peak level at 24hr (104.53±13.07pg/ml) (Figure 2.13B). Serum IL-1β was significantly increased at 5hr (25.31±4.46pg/ml), with levels returning to those of control at 24hr (18.18±2.54pg/ml) (Figure 2.13C). Both the APAP dose response and time course studies showed the typical APAP-associated centrilobular cell loss (Table 2.6 and Table 2.7). At 5hr post dosing, there was no evidence of inflammatory cell recruitment in the liver. This was seen at 24hr with a dose of 530mg/kg, although with variable intensity (slight to moderate), and was represented by the presence of neutrophils within centrilobular areas of hepatocyte loss (Figure 2.10; Table 2.7)

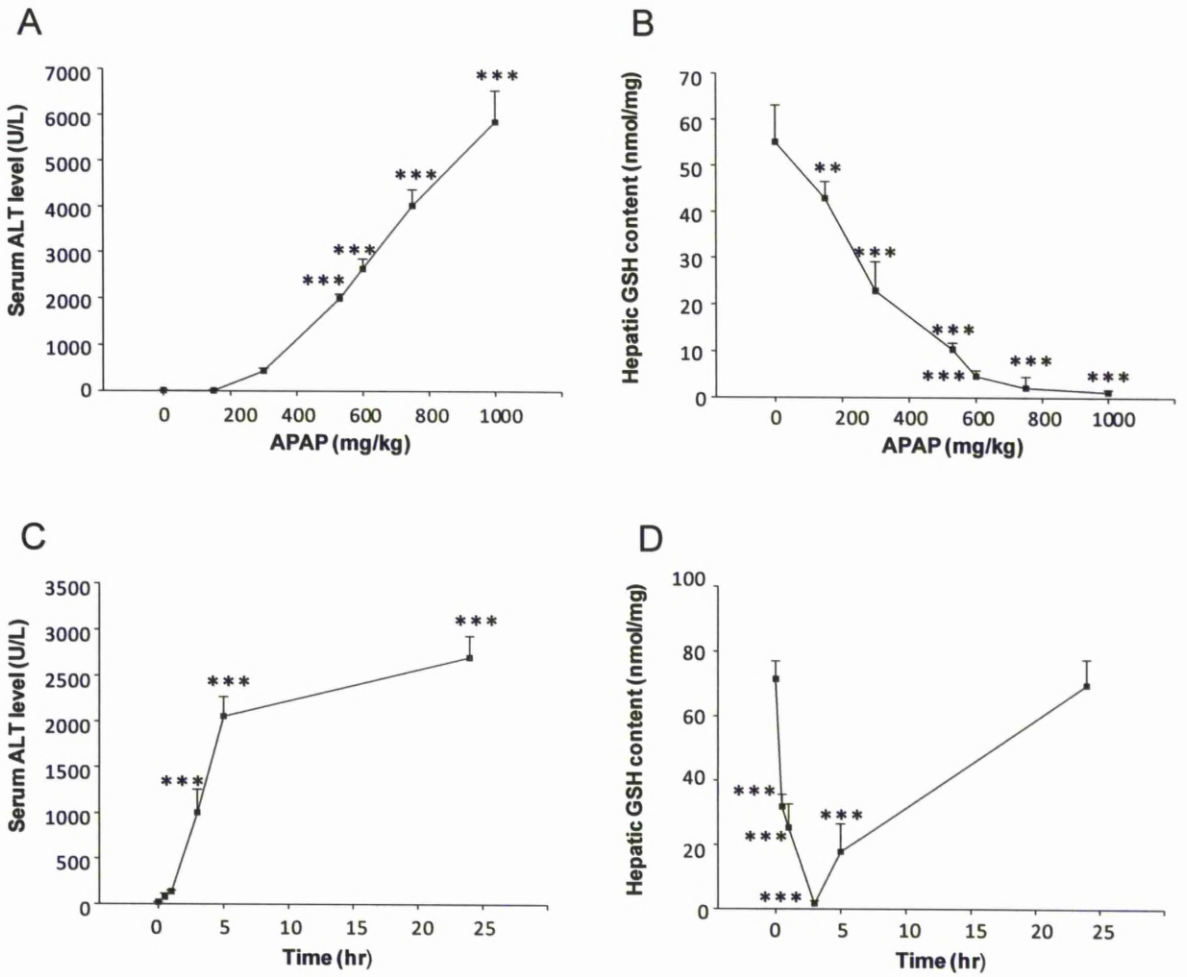

Figure 2.12: APAP-induced hepatotoxicity in male C57BL/6 mice.
The dose effect of APAP (0-1000mg/kg) on serum ALT activity (A) and hepatic GSH content (B) was assessed at 5hr. The time dependent effects of APAP (530mg/kg) on serum ALT activity (C) and hepatic GSH content (D) were assessed over 0-24hr. Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to saline treated controls. $^{**}p < 0.01$ and $^{***}p < 0.001$.

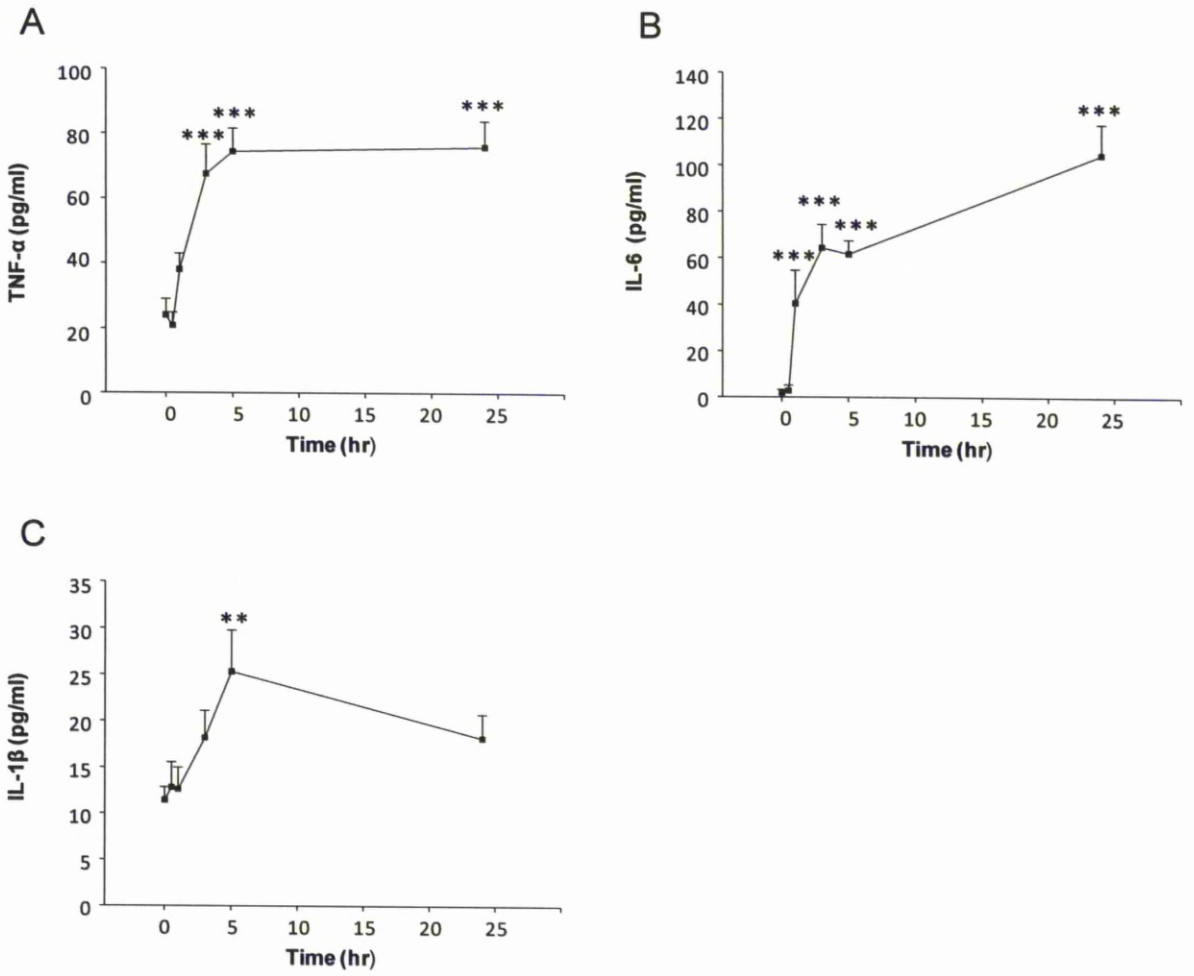

Figure 2.13: Serum cytokine levels during APAP-induced hepatotoxicity in male C57BL/6 mice.

The time dependent effects of APAP (530mg/kg) on serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined in male C57BL/6 mice over 0-24hr. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to saline treated controls. $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 2.6: Histological findings in mice at 5hr after APAP treatment (dose response). The dose effect of APAP (0-1000mg/kg) on male C57BL/6 mouse livers was assessed at 5hr. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed and the range of scores and average score in each of 5 animals per group.

APAP dose	**Score [average]**	**Histological findings at 5hr post dose**
0mg/kg	0	Diffuse glycogen in hepatocytes; no histological abnormality is recognised (NHAIR)
150mg/kg	0	Diffuse glycogen in hepatocytes; NHAIR
300mg/kg	0	Centrilobular hepatocellular glycogen loss (small rim) with occasional hepatocellular hydropic degeneration
530mg/kg	3-4 [3.42]	Hydropic degeneration of remaining cells in areas of cell loss; generally reduced amount of glycogen in hepatocytes in unaffected areas
600mg/kg	3-4 [3.58]	Zones of cell loss surrounded by small rim with loss of glycogen and hydropic degeneration; remaining hepatocytes unaltered with glycogen
750mg/kg	2-4 [3]	Hydropic degeneration of non-necrotic cells surrounding necrosis; some remaining unaltered hepatocytes immediately periportal; no glycogen
1000mg/kg	3-5 [4.14]	Marked hydropic degeneration of remaining hepatocytes in affected areas; variable degree of hydropic degeneration of hepatocytes outside affected areas; no glycogen

Table 2.7: Histological findings in mice after APAP treatment (time course). The time dependent effects of APAP (530mg/kg) were assessed over 0-24hr. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed and the range of scores and average score in each of 4 animals per group.

Time post dosing	Score [average]	Histological findings
0hr	0	Diffuse glycogen in hepatocytes; no histological abnormality is recognised (NHAIR)
1hr	0	Diffuse glycogen in hepatocytes; NHAIR
3hr	1-3 [1.25]	Hydropic degeneration of remaining cells in affected areas; loss of glycogen of hepatocytes surrounding affected areas; no or very occasional apoptotic hepatocytes in affected areas
5hr	1-4 [2.75]	Hydropic degeneration of remaining cells in affected areas; loss of glycogen of hepatocytes surrounding affected areas
24hr	1	Hydropic degeneration and coagulative necrosis of remaining centrilobular hepatocytes; small rim of glycogen loss around affected areas; some centrilobular neutrophils

2.3.6 Effect of fasting on APAP-induced hepatotoxicity and inflammation in C57BL/6 mice

The effects of overnight fasting on APAP-induced hepatotoxicity, metabolism and inflammation were explored in male C57BL/6 mice. Serum ALT activity increased in a time dependent manner compared to control, with a significant increase observed at 3hr (1470.74±345.02U/l), 5hr (2240.83±425.04U/l) and 24hr (3597.04±299.46U/l) (Figure 2.14A). There was a significant decrease in hepatic GSH content observed at 30min post APAP dose (15.29±4.83nmol/mg) which continued to decrease until 3hr where levels reached 5% of control (2.02±0.14nmol/mg) (Figure 2.14B). Hepatic GSH levels began to increase at 5hr and were returning to control levels at 24hr (29.94±3.77nmol/mg). Serum TNF-α was significantly increased from control levels at 3hr (145.32±17.84pg/ml), 5hr (123.94±23.73pg/ml) and 24hr (249.81±39.55pg/ml) post APAP dose (Figure 2.15A). Serum IL-6 levels followed a similar pattern to TNF-α levels and were significantly increased at 3hr

and above (119.67±16.31pg/ml), reaching their peak at 24hr (194.31±35.72pg/ml) (Figure 2.15B). Serum IL-1β was significantly increased at 1hr (38.90±4.56pg/ml), reaching peak levels at 5hr (65.10±9.08pg/ml) with a lower level at 24hr (48.72±6.63pg/ml) (Figure 2.15C). Animals that had been fasted prior to treatment showed complete hepatocellular glycogen depletion (Figure 2.16A). Hepatocellular glycogen was restored after 30min (Figure 2.16B), but appeared to be lost again 1hr post APAP dosing. At 3hr it was again restored which was apparent in treated animals in unaffected areas. Definite histological evidence of APAP-induced centrilobular hepatocyte loss was seen at 5hr post dosing, but there was evidence of hepatocyte damage already at 3hr post dosing, when centrilobular loss of hepatocellular glycogen was observed (Figure 2.17; Table 2.8).

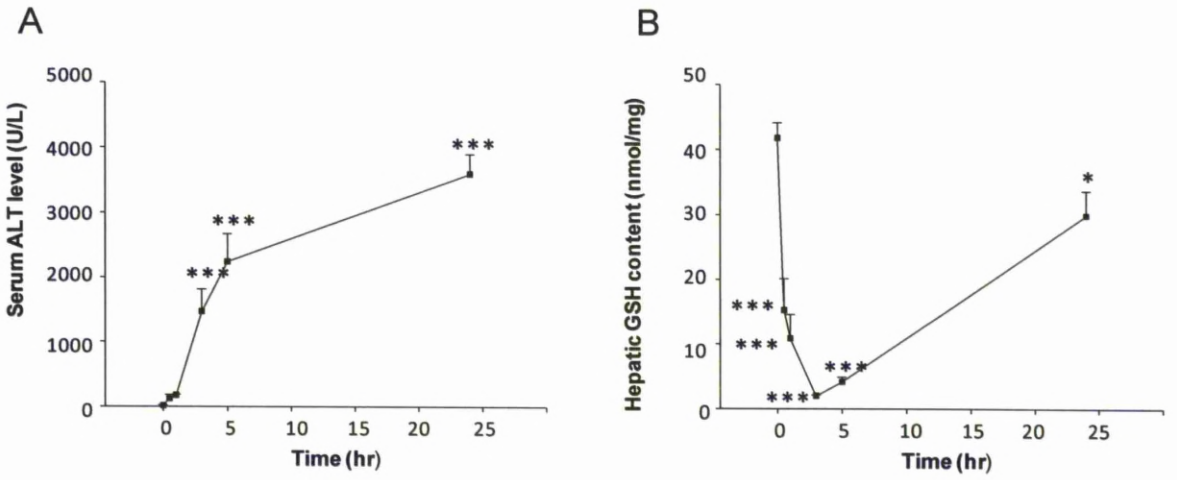

Figure 2.14: APAP-induced hepatotoxicity in fasted male C57BL/6 mice.
The time dependent effects of APAP (530mg/kg) on serum ALT activity (A) and hepatic GSH content (B) were assessed over 0-24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to saline treated controls. $*p < 0.05$ and $***p < 0.001$.

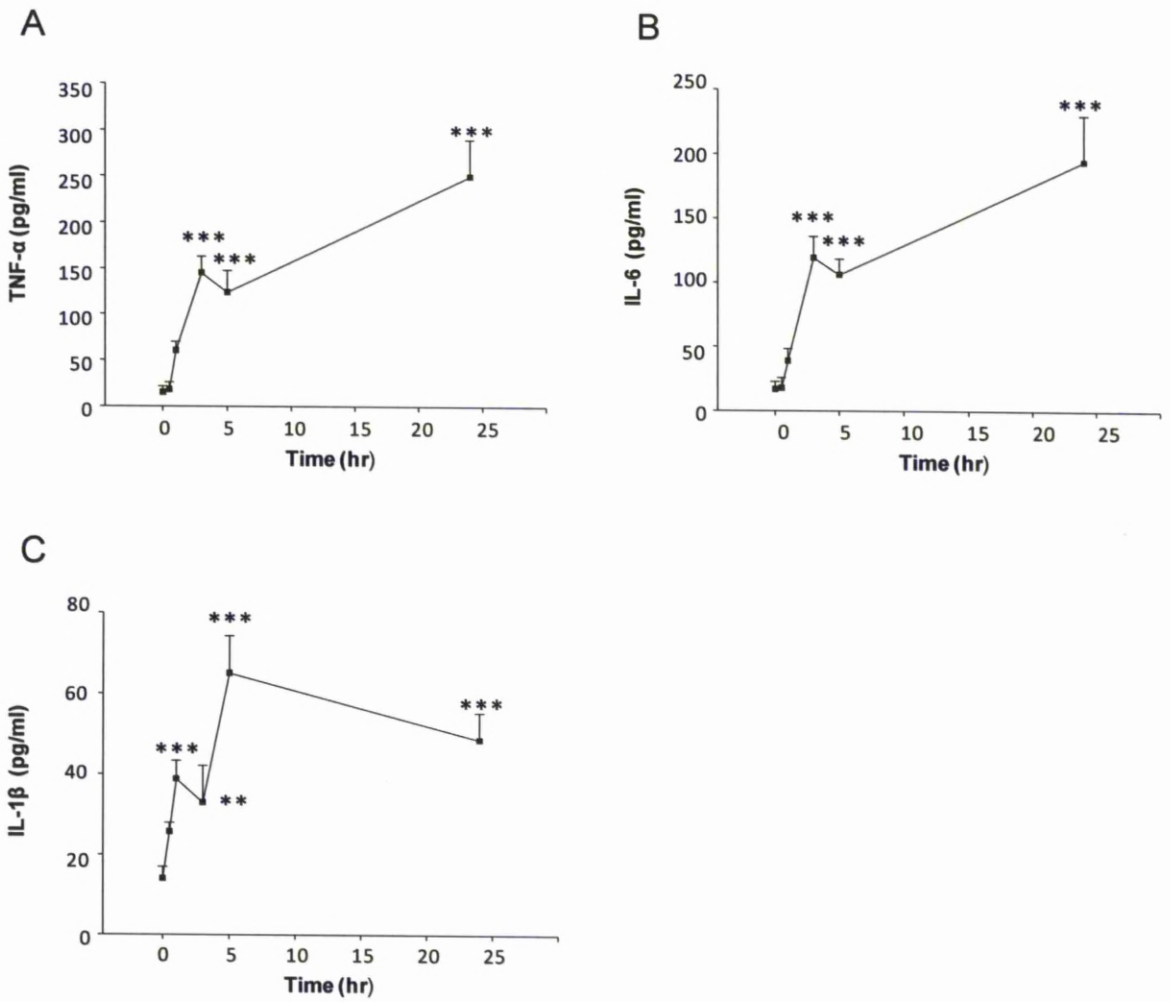

Figure 2.15: Serum cytokine levels during APAP-induced hepatotoxicity in fasted male C57BL/6 mice.

The time dependent effects of APAP (530mg/kg) on serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined in fasted male C57BL/6 mice over 0-24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to saline treated controls. $^{**}p < 0.01$ and $^{***}p < 0.001$.

Table 2.8: Histological findings in fasted mice after APAP treatment (time course). The time dependent effects of APAP (530mg/kg) were assessed over 0-24hr. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed and the range of scores and average score in each of 4 animals per group.

Time post dose	Score [average]	Histological findings
0hr	0	No glycogen (Figure 2.16A); no histological abnormality is recognised (NHAIR)
0.5hr	0	Diffuse glycogen (relatively low amount; Figure 2.16B); one animal with centrilobular loss of glycogen
1hr	0	No glycogen; one animal with centrilobular hydropic degeneration
3hr	0	Extensive centrilobular glycogen loss and hydropic degeneration (Figure 2.17)
5hr	3-4 [3.25]	Hydropic degeneration of remaining hepatocytes in affected areas
24hr	2-3 [2.1]	Hydropic degeneration, coagulative necrosis and apoptosis of remaining hepatocytes in affected areas

NB: Control animals exhibited no hepatocellular glycogen at 0hr. Hepatocellular glycogen was restored from 0.5hr onwards

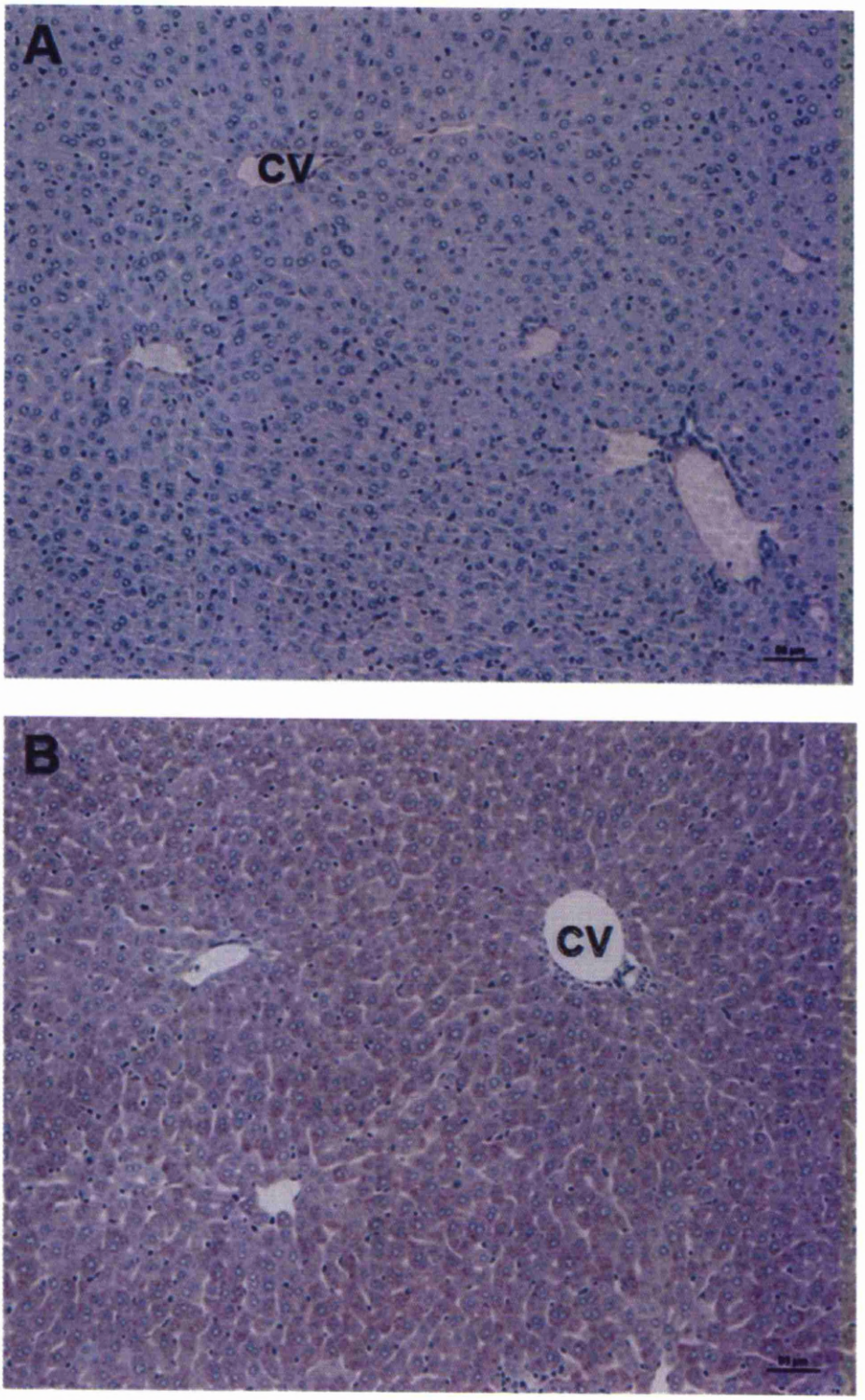

Figure 2.16: Assessment of hepatocellular glycogen content in C57BL/6 mice at 0hr and 0.5hr post APAP treatment (530mg/kg).
(A) At 0hr, no hepatocellular glycogen is observed. (B) At 0.5hr, hepatocellular glycogen is restored, although at generally low level. PAS reaction.

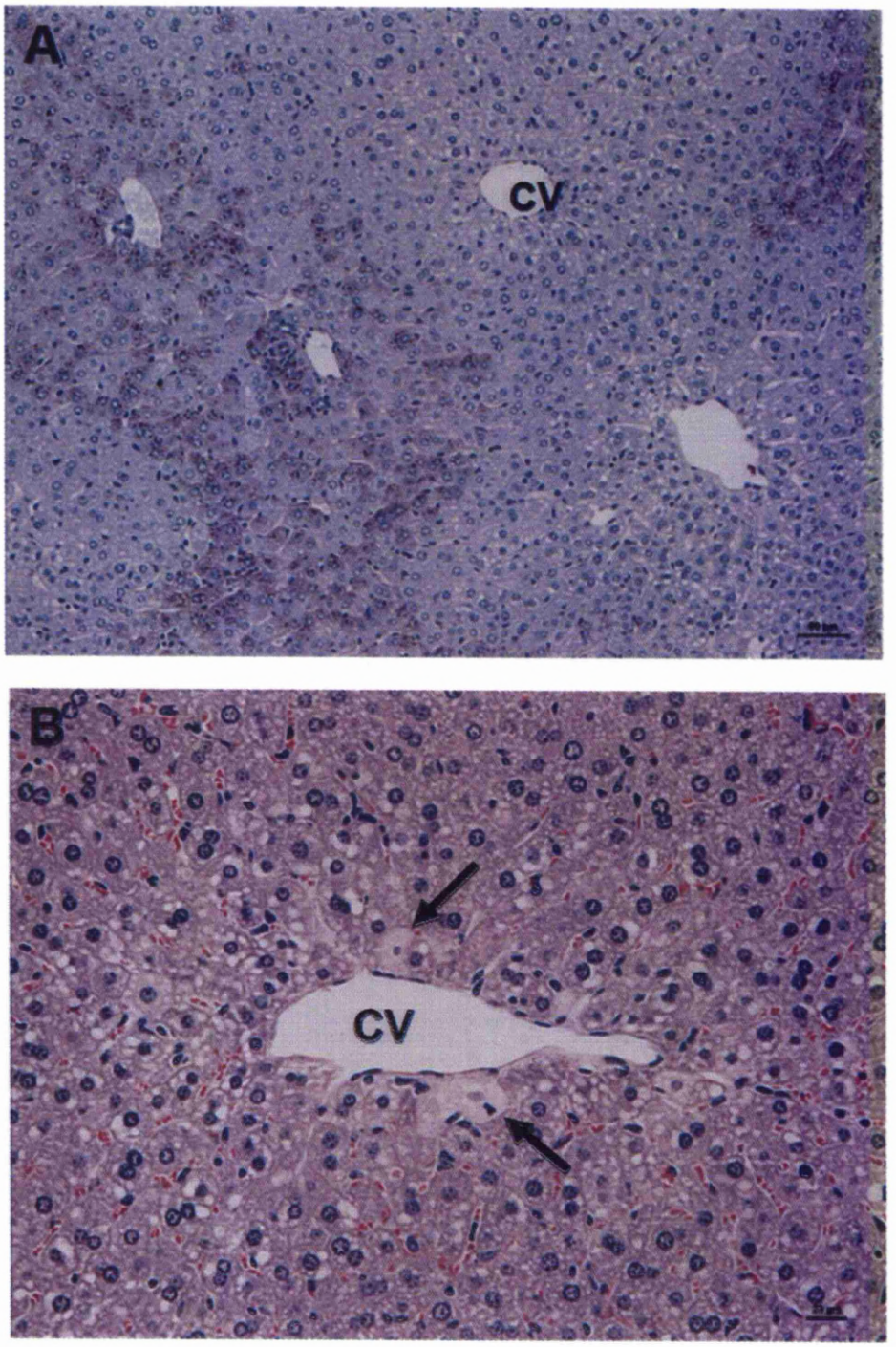

Figure 2.17: Assessment of hepatocellular glycogen content in a C57BL/6 mouse at 3hr post APAP treatment (530mg/kg).

(A) Extensive centrilobular hepatocellular glycogen loss is observed. (B) Glycogen loss is associated with hydropic degeneration of individual centrilobular hepatocytes (arrows), central veins (CV). HE stain.

2.3.7 Comparison of APAP-induced effects on fasted and non-fasted C57BL/6 mice

Following the assessment of APAP hepatotoxicity in fasted C57BL/6 mice, a comparison was made of APAP (530mg/kg) induced changes and basal factors with non-fasted C57BL/6 mice at 5hr and 24hr post APAP dose. Table 2.9 illustrates that serum ALT activity was significantly higher in APAP treated fasted mice at 24hr (3597.04±299.46U/l) than fed mice (2709.49±231.65U/l). Basal levels of hepatic GSH content were higher in fed mice at 5hr (83.85±6.65nmol/mg) than in fasted (38.20±3.09nmol/mg) and also at 24hr. Levels of GSH were also lower in fasted mice at 5hr post APAP dose (4.18±0.70nmol/mg) than in fed (17.78±8.77nmol/mg) and a similar effect was seen at 24hr. Serum levels of TNF-α were significantly higher in fasted mice at 5hr post APAP dose (123.94±23.73pg/ml) than in fed (74.37±7.13pg/ml) as were levels of IL-6 (106.29±12.34pg/ml and 61.69±5.75pg/ml respectively) and IL-1β (65.10±9.08pg/ml and 25.31±4.46pg/ml respectively). Also at 24hr post APAP dose serum levels of TNF-α were significantly higher in fasted mice (249.81±39.55pg/ml) than fed (75.86±7.94pg/ml) as were levels of IL-6 (194.31±35.72pg/ml and 104.53±13.07pg/ml respectively) and IL-1β (48.72±6.63pg/ml and 18.18±2.54pg/ml respectively).

Table 2.9: Summary and comparison of APAP induced effects in fasted and non-fasted male C57BL/6 mice (APAP 530mg/kg; 5hr and 24hr). Data is given as mean (±SD) of 4-5 mice per group. Statistical significance was assigned for non-fasted relative to fasted C57BL/6 mice. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

	Fed		Fasted	
	Control	**APAP**	**Control**	**APAP**
5hr after dose				
Age (weeks)	4-6	4-6	4-6	4-6
Starting body weight (g)	20.27 (1.63)	19.67 (0.36)**	18.01 (1.49)	18.26 (0.41)
Serum ALT (U/l)	14.92 (10.41)	2056.82 (214.47)	11.67 (7.16)	2240.83 (425.04)
Hepatic GSH content (nmol/g)	83.85 (6.65)***	17.78 (8.77)*	38.20 (3.09)	4.18 (0.70)
TNF-α (pg/ml)	3.30 (2.09)	74.37 (7.13)**	-	123.94 (23.73)
IL-6 (pg/ml)	-	61.69 (5.75)**	-	106.29 (12.34)
IL-1β (pg/ml)	-	25.31 (4.46)**	-	65.10 (9.08)
24hr after dose				
Age (weeks)	4-6	4-6	4-6	4-6
Starting body weight (g)	20.25 (1.23) **	21.48 (1.05)***	16.16 (0.69)	15.67 (1.32)
Serum ALT (U/l)	8.49 (7.28)	2709.49 (231.65)**	12.39 (6.81)	3597.04 (299.46)
Hepatic GSH content (nmol/g)	82.26 (5.48)***	69.75 (7.86)***	38.23 (4.60)	29.94 (3.77)
TNF-α (pg/ml)	3.60 (4.33)*	75.86 (7.94)***	20.33 (5.10)	249.81 (39.55)
IL-6 (pg/ml)	4.20 (3.80)	104.53 (13.07)**	24.51 (8.10)	194.31 (35.72)
IL-1β (pg/ml)	-	18.18 (2.54)**	14.35 (3.34)	48.72 (6.63)

2.4 DISCUSSION

Despite intense research, the extent to which the innate immune response contributes to DILI remains disputed. Several different models of DILI in which inflammation has been modulated are being investigated in an attempt to dissect the processes of inflammation from those of direct hepatotoxicity. The use of different strains of mice, species, compounds, and their varying doses and pretreatments in *in vivo* models in the literature, while enabling greater insight into the innate immune and inflammatory responses in DILI, have led to conflicting reports regarding the roles these responses play in the progression of DILI.

The aims of this chapter were therefore to define a set of *in vivo* models of DILI that could produce an inflammatory response which could be assessed and characterised by a chosen set of observable or measurable parameters. The inbred C57BL/6 strain of mice was chosen for the mouse model as it is a common strain used in the literature for APAP and LPS investigations. C57BL/6 mice have been used by many research groups studying the mechanisms of APAP toxicity and are commonly used for the generation and analysis of transgenic and knockout animal models (Chiu et al., 2003; Masubuchi et al., 2003; Liu et al., 2004; Liu et al., 2006; Bourdi et al., 2007; Yee et al., 2007; Zurita et al., 2010). They have also been used to explore the effects of LPS on APAP-induced liver injury (Maddox et al., 2010) and LPS/GalN-induced liver injury (Bahrami et al., 1994; Nowak et al., 2000). LPS and GalN were chosen as model hepatotoxins that can serve as mechanistic models to compare to APAP, both of which trigger an inflammatory response in mice, but with a biological disconnection to APAP since they do not require metabolic activation into a toxic reactive metabolite. To characterise the LPS, GalN and APAP models of hepatotoxicity and inflammation, markers of toxicity and necrosis (serum ALT and histology), APAP-mediated metabolism (hepatic GSH content) and inflammation (neutrophil accumulation and serum cytokines TNF-α, IL-6 and IL-1β) were evaluated in a time and dose dependent manner.

LPS is a bacterial endotoxin that can trigger a potent inflammatory response through numerous inflammatory pathways via activation of TLR4. These include intracellular signalling pathways and transcription factors such as p38 and NF-κB and inflammatory mediators such as cytokines and chemokines (Deng et al., 2009). The release of these

mediators can lead to the activation and hepatic infiltration of innate immune cells such as neutrophils, which propagate tissue damage. In C57BL/6 mice, no hepatotoxicity as determined by ALT activity or effect on total hepatic GSH content was observed across a dose response or time course with LPS (Figure 2.1). These results were consistent with other published findings (Maddox et al., 2010). At 24hr with a dose of 5mg/kg the serum protein levels of the cytokines TNF-α, IL-6 and IL-1β were significantly increased (Figure 2.2). These results confirm that administration of LPS to mice at the dose used in this study produces a systemic inflammatory response, as evidenced by the increase in serum levels of TNF-α, IL-6 and IL-1β. While this appeared to induce some influx of leukocytes into the liver, it was not associated with overt liver damage, since there was no evidence of parenchymal cell death. Despite there being no evidence of damaged hepatocytes, TNF-α and IL-6 has been observed in hepatocytes of mice treated with LPS (5mg/kg) at 24hr by immunohistochemical analysis (data not shown, personal communication with Prof Anja Kipar), suggesting that the cytokines detected in the serum are not only from activated leukocytes alone. This model therefore is characterised by a potent inflammatory response with no hepatotoxicity and is suitable as a positive control for inflammation.

GalN is a hepatotoxin frequently used to induce experimental liver injury (Keppler et al., 1968). Excessive doses of GalN induce fatal liver injury that histologically resembles human hepatic failure, while transient acute liver injury is observed at lower doses (Keppler et al., 1968). High doses of GalN are known to cause both hepatic necrosis by UTP depletion, leading to inhibition of RNA synthesis (Decker and Keppler, 1974) and apoptosis in rats and mice (Tsutsui et al., 1997; Itokazu et al., 1999; Stachlewitz et al., 1999; Gujral et al., 2003a). GalN was chosen as a model hepatotoxin because the mechanism of toxicity is different to APAP while GalN is known to produce an inflammatory response. The inflammatory response in the liver resembles the reaction seen in viral hepatitis (Medline et al., 1970), also antibodies to TNF-α have been shown to protect against GalN-induced hepatotoxicity in rats (Stachlewitz et al., 1999). The aim was to develop a GalN-induced hepatotoxicity model of inflammation in mice. In C57BL/6 mice, GalN hepatotoxicity (as determined by serum ALT activity) showed a dose dependent effect without any significant effect on total hepatic GSH content at 5hr (Figure 2.3). The levels of serum ALT were significantly raised at dose levels of 600mg/kg and 800mg/kg. To be able to characterise GalN-induced hepatotoxicity fully at a detectable level, a more detailed dose response study and time course were assessed. The

dose response measurements were extended from 5hr to 24hr to allow time for toxicity to occur. These results showed that serum ALT activity significantly increased in a dose dependent manner above the threshold of toxicity at doses of 600-1500mg/kg (Figure 2.4). Increasing the dose still had no significant effect on hepatic GSH content which is consistent with evidence that GalN is not metabolised into a toxic reactive metabolite therefore will not form a GSH conjugate level in the liver (Maley et al., 1968). GalN hepatotoxicity also showed time dependence as ALT activities were significantly raised at 5hr and 24hr and GSH content was not affected. From the dose and time course experiments the level of serum cytokines were assessed at 24hr at a dose of 800mg/kg. Levels of TNF-α, IL-6 and IL-1β were all significantly increased although one point to notice was that the levels were markedly lower in comparison to levels observed with LPS. The results suggest that this model is characterised by moderate hepatotoxicity and a moderate inflammatory response as assessed by cytokine release. The histological assessment confirmed GalN-associated hepatocyte necrosis and apoptosis at higher doses, with a mixed cellular inflammatory infiltrate.

The secondary aim was to characterise the neutrophilic inflammatory response after treatment of C57BL/6 mice with APAP. To define a degree of hepatotoxicity which may cause neutrophil infiltration a range of APAP doses (300mg/kg, 530mg/kg or 750mg/kg) were administered to male C57BL/6 mice (Figure 2.6). In administering a high dose, the hypothesis that extensive hepatocellular necrosis would lead to infiltration and accumulation of immune cells was being tested. Figure 2.8 illustrates that the survival of mice after exposure to APAP was significantly decreased in the treatment groups of the high (750mg/kg) dose at 24hr compared to saline treated and low-dose (300mg/kg) APAP which had 100% survival. Mice receiving 750mg/kg survived up until 8-11hr with none lasting to 24 hrs. The 530mg/kg dosed group had only a couple of mortalities after the 18hr mark, with the majority surviving the 24hr period, despite there being evidence of toxicity. Toxicity at 24hr was dependent upon dose when assessed by serum ALT activity which was significant from control levels at doses above 530mg/kg (Figure 2.6). At the equivalent time point (24hr), the histological assessment of the liver did not identify a significant inflammatory cell influx and the hepatic GSH content was not significantly different from control levels which suggested that a physiological adaptation or regeneration had occurred within the liver, although the level of ALT activity was still high.

The APAP model of hepatotoxicity and inflammation was further characterised by a detailed dose response and time course investigation in male C57BL/6 mice as illustrated by Figure 2.9. APAP induced a dose dependent increase in hepatotoxicity as seen by an increase in serum ALT activity which was first significant at dose levels above 530mg/kg at 5hr compared to control levels. Hepatic GSH depletion occurred in a dose dependent manner starting at 150mg/kg. Hepatotoxicity due to APAP was observed as early as 3hr post APAP dose (530mg/kg) by serum ALT activity, which was preceded by depletion in hepatic GSH content first significant from control at 0.5hr post APAP dose (530mg/kg). These results confirm that in this model, GSH depletion is a prerequisite for the onset of toxicity following APAP overdose (Davis et al., 1974). To determine the time frame of the onset of inflammation in this model the serum protein levels of the cytokines TNF-α, IL-6 and IL-1β were measured in parallel to toxicity. The levels of each cytokine appeared to mimic the pattern of serum ALT activity over the time course with levels of TNF-α and IL-6 reaching a peak at 24hr post APAP dose (530mg/kg) (Figure 2.10). IL-1β diverged from this pattern slightly in that levels peaked at 5hr post APAP (530mg/kg) and began to decrease at 24hr. At 24hr post dosing, the histological assessment identified ongoing centrilobular hepatocyte necrosis and apoptosis as well as some recruitment of neutrophils into the affected areas. The latter was not observed at an earlier time point.

Fasting is used in pharmacological and toxicological experiments to assure more uniform drug absorption (Strubelt et al., 1981). Overnight fasting of animals has been commonly used in studies of APAP-induced hepatotoxicity as fasting reduces hepatic GSH content and reduces its diurnal variation (Jaeschke et al., 1985), enabling more uniform toxicity from moderate doses. Recent reports demonstrated that overnight fasting of mice can prevent APAP-induced apoptosis and lead to hepatocyte death almost exclusively by necrosis (Antoine et al., 2010). Evidence suggests that a biological consequence of fasting is depletion of hepatic glycogen stores which is used to generate cellular ATP (Strubelt et al., 1981). Depletion of hepatic ATP levels can lead to a reduction of apoptotic cell death and switch to necrosis (Kim et al., 2003). Antoine et al. (2010) also demonstrated that infiltration of neutrophils into the liver was seen in fasted but not in fed CD-1 mice after APAP treatment. This was also supported by an increase in serum TNF-α levels and total HMGB1. It was concluded that reduced ATP levels in hepatocytes of fasted animals prevents apoptosis and promotes necrotic cell death which leads to the release of total HMGB1, which triggers

recruitment of inflammatory cells into the liver and formation of serum cytokines. The purpose of the next study was to investigate the impact of overnight fasting on APAP-induced hepatotoxicity and inflammation in C57BL/6 mice and assess if this was a more suitable model for inflammation than the previously characterised fed model. Similar in pattern to the fed model, APAP caused a time dependent elevation in hepatotoxicity as assessed by serum ALT activity, which was first significant at 3hr and continued to increase at the further time points of 5hr and 24hr (Figure 2.11). Hepatic GSH depletion preceded the increase in ALT at 0.5hr and began to return to control levels at 24hr. The serum levels of TNF-α and IL-6 increased significantly at 3hr post APAP dose (530mg/kg) and were preceded by a significant increase in IL-1β at 1hr (Figure 2.12). TNF-α and IL-6 both reached peak concentrations at 24hr post APAP, whereas IL-1β began to decrease nonetheless. The histological examination did not identify significant inflammatory cell influx into the liver at any time point.

Table 2.9 illustrates the comparison between the fasted and non-fasted C57BL/6 models of APAP-induced hepatotoxicity and inflammation. Serum ALT activity was significantly higher in fasted mice than fed at 24hr after APAP dose (530mg/kg). This result is supported by evidence in the literature that fasting of mice prior to dosing can increase the levels of hepatic necrosis and worsen liver injury in animal models (Strubelt et al., 1981). Basel control levels of hepatic GSH content in fed C57BL/6 mice were higher than in fasted mice at 5hr and 24hr. This may account for the increased sensitivity of fasted mice to the toxic effects of APAP as seen by serum ALT activity as decreased hepatic GSH content may result in a lesser detoxification of APAP and therefore a higher amount of the reactive metabolite NAPQI, enhancing the hepatotoxicity of APAP. Overnight fasting of mice also had the effect of increasing the serum protein concentrations of cytokines TNF-α, IL-6 and IL-1β at both 5hr and 24hr post APAP (530mg/kg) dose compared to fed mice. One reason for this could be a change in the predominant form of cell death. Fasting may lead to hepatocyte death almost exclusively by necrosis due to evidence that fasting leads to depletion of intracellular ATP (Lee et al., 1988), inhibiting the energy-dependent process of apoptosis. Increasing the level of necrosis would lead to the release of more inflammatory signals and mediators, promoting a greater inflammatory response. Further investigations are required to fully determine why fasting prior to experimental DILI elicits a greater inflammatory response in animals.

The aims of the studies presented in this chapter were to utilise LPS, GalN and APAP as model hepatotoxins to enable the characterisation of the inflammatory response in *in vivo* models of hepatotoxicity. Defining the toxic and inflammatory parameters of these models will enable the investigation of various inflammatory pathways during DILI and their contribution to the progression of liver injury. Modulation of these pathways can help examine the overall effect on DILI by apportioning the level of toxicity caused by inflammation or the direct deleterious effects of the model hepatotoxins. As shown with these studies, the three model agents and the dietary conditions of the C57BL/6 strain of mice have produced models of varying levels of toxicity and inflammation. Each of the animal models are equally valid and the variation of each with regards to mechanisms of toxicity allow a more complete exploration and determination of possible inflammatory mechanisms contributing to the overall pathophysiology. With these defined and characterised models, the next course of action would be to investigate the innate immune and inflammatory responses in these models by using pharmacological agents that modulate the mechanism of inflammation.

CHAPTER THREE

INVESTIGATION OF THE EFFECT OF DMSO AND ASPIRIN ON DRUG-INDUCED HEPATOTOXICITY AND MODULATION OF THE INFLAMMATORY RESPONSE

CONTENTS

3.1 INTRODUCTION

The mechanisms of APAP hepatotoxicity are well defined and characterised in experimental animals and humans (Mitchell et al., 1973). In addition to the intracellular biochemical events there is growing evidence that suggests that the innate immune system can contribute to the severity and progression of APAP-induced liver injury through the production of cytokines and recruitment of inflammatory cells into the liver downstream of bioactivation (Blazka et al., 1995; Laskin et al., 2001; Liu et al., 2004). The generation of inflammatory mediators such as cytokines, chemokines, reactive oxygen and nitrogen species released by inflammatory cells have been implicated in APAP hepatotoxicity by exacerbating cell damage as a result of initiating an overly aggressive inflammatory process (Luster et al., 2001; Liu et al., 2004). It is therefore increasingly important to understand the mechanisms by which cells of the innate immune system are activated and recruited to the liver during DILI.

The formation and release of proinflammatory cytokines such as TNF-α and IL-1β during APAP-induced liver injury have been extensively investigated (Blazka et al., 1995; Lawson et al., 2000) and the results presented in Chapter 2 provide supporting evidence of the presence of an inflammatory response in mice after APAP overdose. However, only recently have the initiating mechanisms of an inflammatory response been identified. The direct effect of APAP on hepatocytes may be an initiating event for an immune response as necrotic cells release endogenous molecules that alert the innate immune system of the danger associated with tissue damage. These molecules collectively termed damage-associated molecular patterns (DAMPs) include HMGB1, heat shock proteins (HSPs) and DNA fragments (Bianchi et al., 2007) and act as danger signals by activating a variety of pattern recognition receptors including TLRs, RAGE and NLRs. As a result, necrotic cells and several danger signals directly trigger proinflammatory responses.

The liver contains a large number of various types of innate immune cells such as Kupffer cells, NK cells and NKT cells. Aside from the resident innate immune cells the release of DAMPs and formation of cytokines during the inflammatory response lead to hepatic infiltration and activation of neutrophils and monocytes in response to tissue injury (Lawson

et al., 2000; Scaffidi et al., 2002). Depletion of NK and NKT cells in the liver was reported to protect mice from APAP hepatotoxicity (Liu et al., 2004). Depletion of these cells substantially reduced the formation of cytokines and chemokines, notably IFN-γ after APAP overdose and associated neutrophil accumulation in the NK/NKT cell depleted mice. There are however, conflicting reports concerning the role of NK cells and NKT cells in APAP-induced liver injury. One study provided evidence that NKT and NK cells only contributed to liver injury when dimethyl sulfoxide (DMSO) was used to solubilise APAP (Masson et al., 2008). The report revealed that DMSO, both alone and with APAP, led to the activation of hepatic NKT and NK cells as shown by higher NKT cell numbers and that the effect of NKT cells in the exacerbation of APAP-induced liver injury was due to high levels of DMSO (Masson et al., 2008). The depletion of these cell types in mice did not alter APAP-induced injury unless the cells had been pre-activated with DMSO.

Neutrophils have also been implicated as having an active role in the pathogenesis of APAP hepatotoxicity (Ishida et al., 2006; Liu et al., 2006). Depletion of neutrophils with an anti-granulocyte antibody significantly protected mice against APAP-induced liver injury as evidenced by reduced serum ALT levels and improved mice survival. The involvement of neutrophils was also implied in a recent study that suggested APAP-induced hepatotoxicity is partially mediated by IL-1β, which can activate and recruit neutrophils, exacerbating injury (Imaeda et al., 2009). It was shown in this study that stimulation of TLR9 by DNA fragments during APAP-induced hepatotoxicity lead to the transcriptional activation of the IL-1β gene resulting in formation of pro-IL-1β. The pro-form of IL-1β is cleaved by activated caspase-1, whose activation is regulated by the Nalp3 inflammasome. Imaeda et al (2009) showed that APAP-induced liver injury was reduced in gene knockout mice of each individual component of the inflammasome and also by inhibition of inflammasome activation by aspirin. This suggested an important role of inflammasome activation of IL-1β formation in APAP-induced hepatotoxicity and potential therapeutic strategy to treat APAP hepatotoxicity. However, there are conflicting views regarding the importance of the inflammasome and the role of neutrophils. IL-1β signalling does not directly induce cell death *in vivo* (Sims and Smith, 2010) but causes liver injury through activation of inflammatory cells such as neutrophils (Bajt et al., 2001). However, conflicting reports suggest neutrophils are unlikely to cause injury after APAP overdose (Bauer et al., 2000; Cover et al., 2006; Lawson et al., 2000). One study suggested that although the numbers of neutrophils were reported to be

increased in the liver from APAP-challenged animals, neutrophils did not contribute to the initiation and progression of APAP-induced liver injury. The inflammation observed after overdose was stated as not being severe enough to cause additional damage (Lawson et al., 2000).

The overall extent of the contribution of the innate immune system to the progression of APAP-induced liver injury requires further investigation. The aims of the studies within this chapter were to identify the role DMSO may play in APAP hepatotoxicity by modulation of hepatic immune cell accumulation and whether in our animal model of APAP-induced liver injury the addition of DMSO could potentiate toxicity by initiating a greater inflammatory response. Secondly, one objective of this investigation was to further evaluate the contribution of the immune system in the pathogenesis of DILI by using aspirin as a pharmacological intervention of the Nalp3 inflammasome in our animal models characterised in Chapter 2.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Infinity ALT liquid kits were purchased from ALPHA Laboratories (Eastleigh, U.K). Unless otherwise stated, all other chemicals and materials were purchased from Sigma-Aldrich (Poole, U.K).

3.2.2 Experimental animals

The protocols described were in accordance with criteria outlined in a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Animals were purchased from Charles River laboratories and had a 5 day acclimatisation period prior to experimentation. Animals were also maintained in a 12hr light/dark cycle with free access to food and water.

3.2.3 Animal dosing regime

Male C57BL/6 (18-23g) mice were either fasted overnight with free access to water or had free access to food and water prior to experimentation. Mice received an *i.p* injection of APAP (530mg/kg) with a simultaneous *i.p* injection of DMSO (0-1ml/kg) or 1-4hr after APAP administration. Control animals received either 0.9% saline or solvent control in 0.9% saline as appropriate. Treatment groups contained 4-5 individual animals. Additionally, some mice were given aspirin via drinking water (0.06mg/ml, 0.3mg/ml, 0.6mg/ml or 1mg/ml) for three days and fasted overnight prior to either LPS (5mg/kg; *i.p*), GalN (800mg/kg; *i.p*) or APAP (530mg/kg; *i.p*).

3.2.4 Assessment of hepatotoxicity in male C57BL/6 mice

Animals were sacrificed by CO_2 inhalation and cervical dislocation and blood was collected by cardiac puncture. Hepatotoxicity was assessed by serum ALT activity and histology as described in Chapter 2.

3.2.5 Determination of hepatic glutathione levels in C57BL/6 mouse whole liver during drug-induced hepatotoxicity

Total hepatic GSH levels were determined as described previously in Chapter 2. Levels of GSH were normalised to hepatic protein content using Bio-Rad protein assay reagent according to the manufacturer's instructions.

3.2.6 Histological examination of C57BL/6 mouse liver

Hepatotoxicity and leukocyte infiltration was assessed as part of the histological examination of HE-stained sections as described in Chapter 2. The assessment was undertaken by Prof A Kipar, Veterinary Pathology, School of Veterinary Science, University of Liverpool.

3.2.7 Measurement of cytokines in serum from animals dosed with LPS, GalN and APAP using Luminex Analysis

Serum was collected from control and treated animals. Concentrations of IL-1β, IL-6 and TNF-α were measured using the Bio-Plex Assay kit (Bio-Rad Laboratories, Inc) as described in Chapter 2. The lower range of the assay is <5.8 pg/ml for TNF-α, <0.74pg/ml for IL-6 and <10.36pg/ml for IL-1β. Levels below these values were assigned a value of zero.

3.2.8 Measurement of HMGB1 in serum from animals dosed with LPS

HMGB1 measurements were carried out using an ELISA according to the manufacturer's instructions. A chicken anti-HMGB1 antibody was used as a catcher antibody (1μg/well compared to known standards using 3,3',5,5'-tetramethyl-benzidine turnover by a peroxidise-linked anti-HMGB1 antibody. The limit of detection was 0.1ng/ml.

3.2.9 Statistical analysis

All results are expressed as mean ± standard deviation (S.D). Values to be compared were analysed for non-normality using a Shapiro-Wilk test. The unpaired t-test was used when non-normality was indicated. A Mann-Whitney U test was used for non-parametric data. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a *post* hoc Bonferroni test. All calculations were performed using SPSS statistical software, results were considered significant when $p<0.05$.

3.3 RESULTS

3.3.1 Investigation into the effect of DMSO on APAP-induced hepatotoxicity

To investigate the effect of DMSO in APAP-induced hepatotoxicity by inhibition of APAP bioactivation or immune cell infiltration, mice were either simultaneously co-administered DMSO (1ml/kg) or administered DMSO 2hr after APAP (530mg/kg) for 24hr (Figure 3.1). Significant toxicity as determined by serum ALT activity was observed in APAP treated mice (2468.65±380.78U/l) and DMSO 2hr post-APAP (2001.55±228.45U/l) compared to solvent control (Figure 3.1A). No toxicity was evident when DMSO was dosed simultaneously with APAP (27.68±4.85U/l). At 24hr there was significant depletion of hepatic GSH content observed between control and APAP dosed groups (77.55±4.47nmol/mg and 54.09±8.28nmol/mg respectively) (Figure 3.1B). GSH returned to control levels when dosed with APAP and DMSO concurrently (69.95±7.38nmol/mg) but was significantly lower than control when DMSO was dosed 2hr post-APAP (63.97±2.66nmol/mg). DMSO induced a dose dependent decrease in serum ALT activity at 5hr compared to solvent control (Figure 3.2A). There was a significant decrease in toxicity in groups with DMSO levels greater than 0.1ml/kg (1348.27±325.14U/l) when compared to groups treated with APAP alone (2116.36±193.40U/l). There was significant toxicity in all DMSO and APAP treated groups compared to DMSO treated alone (15.28±5.13U/l). An increase in hepatic GSH levels was observed, notably from DMSO levels greater than 0.1ml/kg (23.04±7.04nmol/mg), with a significant increase at 0.5ml/kg (28.85±9.51nmol/mg) and above (Figure 3.2B). DMSO inhibited APAP-induced toxicity in a time dependent manner. When administered concurrently with APAP, DMSO completely abolished toxicity (45.83±9.11U/l) compared to APAP alone (1863.93±178.34U/l). DMSO administered 1hr post APAP dose also negated toxicity (136.28±18.04U/l), however when administered 2hr and 4hr post APAP dose there was a significant increase in ALT activity (361.02±119.46U/l and 1277.07±230.74U/l respectively) (Figure 3.3A). APAP induced a significant decrease in hepatic GSH content (8.42±2.37nmol/mg) compared to control. DMSO administered concurrently with APAP or 1hr post APAP dose increased GSH content by around 52% compared to APAP alone (33.15±3.12nmol/mg and 32.21±1.89nmol/mg respectively) (Figure 3.3B). DMSO administrated 2hr and 4hr post APAP did not lead to an increase in GSH content compared to APAP alone.

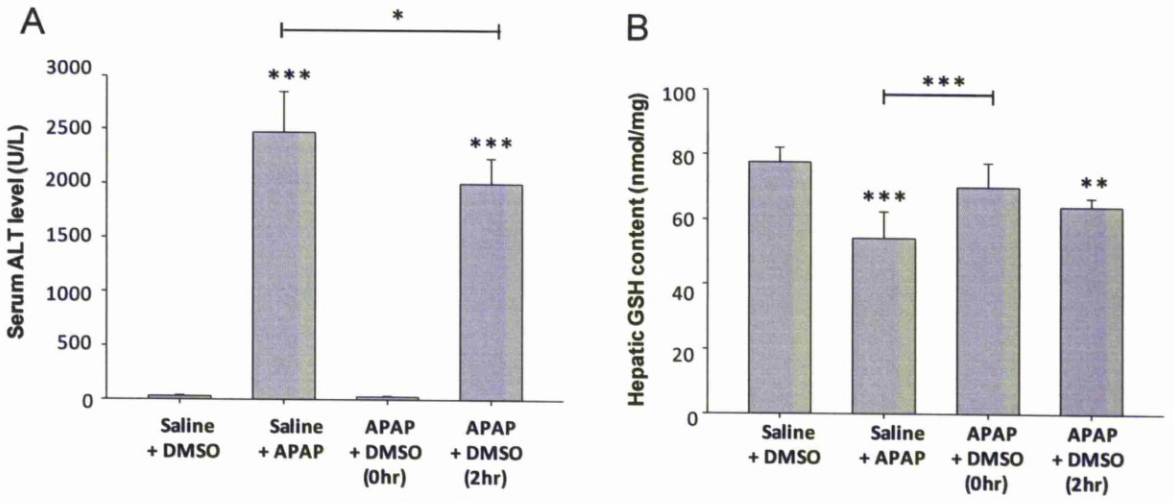

Figure 3.1: The effect of DMSO on APAP-induced hepatotoxicity.
Male C57BL/6 mice were administered APAP (530mg/kg; 24hr) ± DMSO (1mg/ml; 0-2hr) and the effect on APAP induced serum ALT levels (A) and hepatic GSH content (B) was observed in the same group of mice. Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to solvent treated controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The histological examination identified the typical APAP-induced changes in APAP-treated animals. These were completely lacking in animals co-treated with DMSO, whereas they were still present when DMSO was administered 2hr post APAP treatment. The degree and distribution of centrilobular hepatocyte loss was highly variable in these animals (Table 3.1).

Table 3.1: Histological findings after DMSO and APAP treatment. Male C57BL/6 mice were administered APAP (530mg/kg; 24hr) ± DMSO (1mg/ml; 0-2hr) or DMSO alone. Animals were examined 24hr post treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 5 animals per group.

Treatment	Score [average]	Histological findings
Saline + DMSO	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
APAP	1-4 [2.2]	Centrilobular, mainly coagulative necrosis; highly variable degree; sometimes with neutrophil aggregates in centre
APAP + DMSO 0hr	0	Diffuse glycogen; NHAIR
APAP + DMSO 2hr	1-4 [1.9]	Highly variable intensity of cell loss

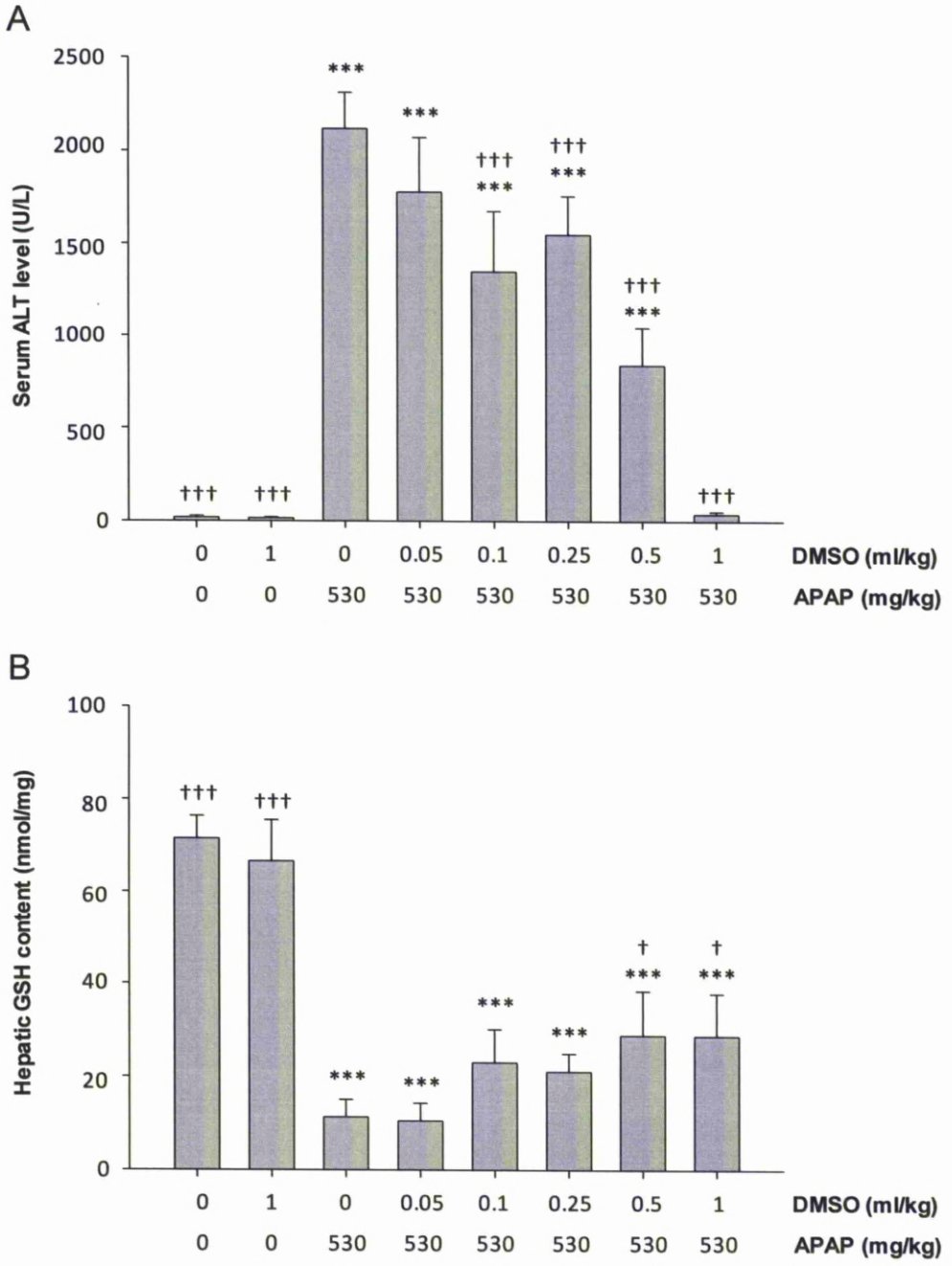

Figure 3.2: Dose dependent effect of DMSO on APAP-induced GSH depletion and toxicity.

Male C57BL/6 mice were administered APAP (530mg/kg; 5hr) simultaneously with DMSO (0-1ml/kg) and the effect on APAP induced serum ALT levels (A, U/l) and hepatic GSH content (B, nmol/mg) was observed in the same group of mice. Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned to groups with values that are higher or lower when compared to DMSO treated controls ***$p < 0.001$ or compared to APAP alone (†$p < 0.05$, †††$p < 0.001$).

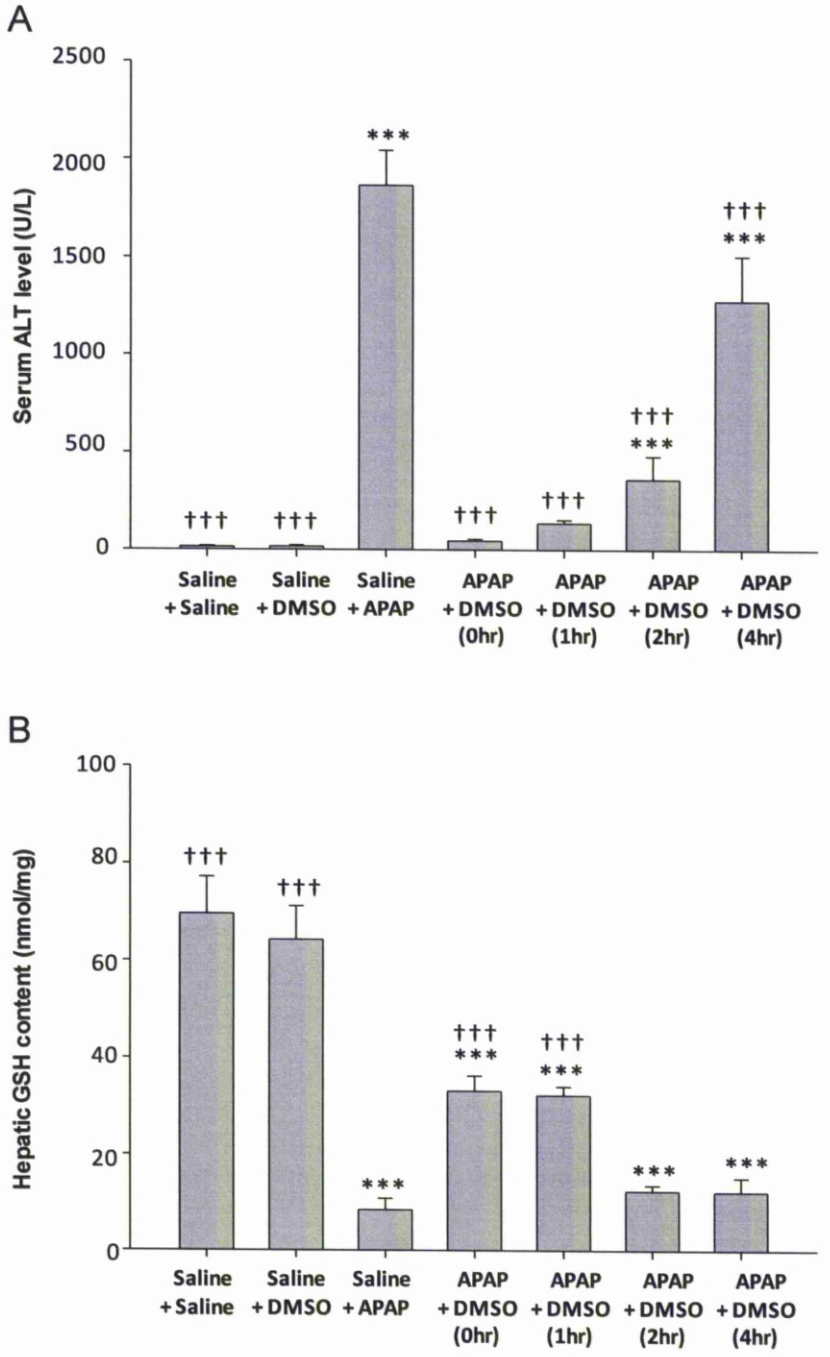

Figure 3.3: Time course of the effect of DMSO on APAP-induced GSH depletion and toxicity.

Male C57BL/6 mice were administered APAP (530mg/kg; 5hr) and DMSO (1ml/kg; 0-4hr post-APAP) and the effect on APAP induced serum ALT levels (A) and hepatic GSH content (B) was observed in the same group of mice. Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to groups with values that are higher or lower when compared to DMSO treated controls (***$p < 0.001$) or compared to APAP alone (†††$p < 0.001$).

3.3.2 Investigation into the effect of aspirin on LPS-induced inflammation

It was hypothesised that aspirin is capable of modulating inflammation by inhibiting the Nalp3 inflammasome and NF-κB activation. As it was reported that pretreatment with aspirin in the drinking water reduced APAP-induced liver injury (Imaeda et al., 2009), the effects of aspirin on the modulation of inflammation were investigated in the LPS mouse model characterised in Chapter 2. This study was repeated with the aspirin dose previously published (0.06mg/ml in drinking water; Imaeda et al., 2009). Aspirin in the drinking water caused no toxicity as assessed by serum ALT activity (48.10±15.93U/l) (Figure 3.4A). LPS administered alone or with aspirin did not cause toxicity (44.61±18.71U/l and 40.44±17.33U/l respectively). Aspirin pretreatment did not alter hepatic GSH content compared to control (79.34±8.19nmol/mg), nor did LPS alone or with aspirin (72.13±2.84nmol/mg and 70.18±12.06nmol/mg respectively) (Figure 3.4B).

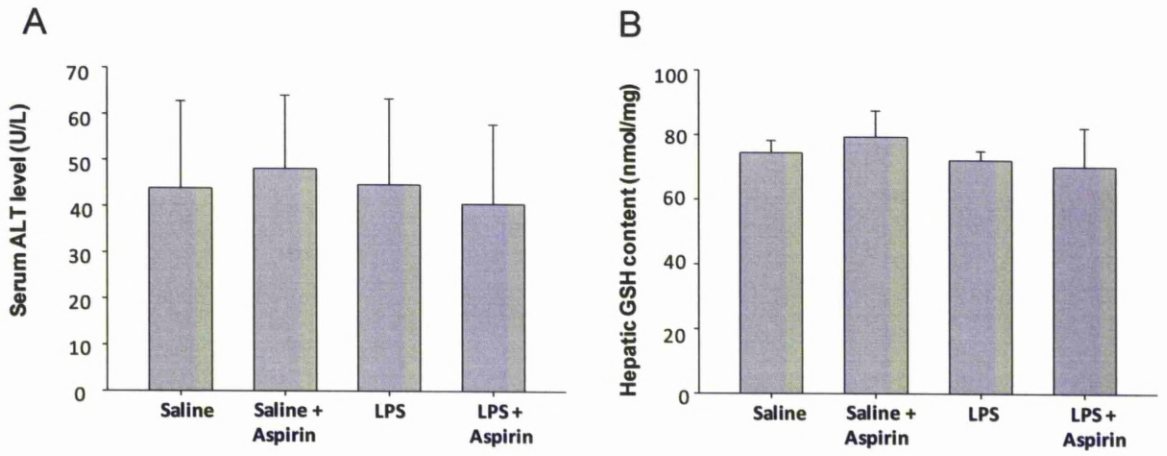

Figure 3.4: The effect of aspirin on LPS-induced hepatotoxicity in male C57BL/6 mice. Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice pretreated with or without aspirin (0.06mg/ml) in drinking water for 3 days before administration of LPS (5mg/kg; 24hr). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin.

Aspirin pretreatment (0.06mg/ml) caused no inflammation as assessed by cytokine and HMGB1 release (Figure 3.5). LPS (5mg/kg) caused a significant increase in the serum level of TNF-α at 24hr (540.95±71.24pg/ml) (Figure 3.5A). IL-6 also increased at 24hr after LPS (1575.35±284.73pg/ml) (Figure 3.5B) as well as levels of IL-1β (238.67±23.13) (Figure 3.5C). Serum levels of HMGB1 were also significantly increased at 24hr after LPS (1093.36±338.50ng/ml) (Figure 3.5D). Pretreatment with aspirin (0.06mg/ml) did not significantly reduce LPS-induced cytokine or HMGB1 release.

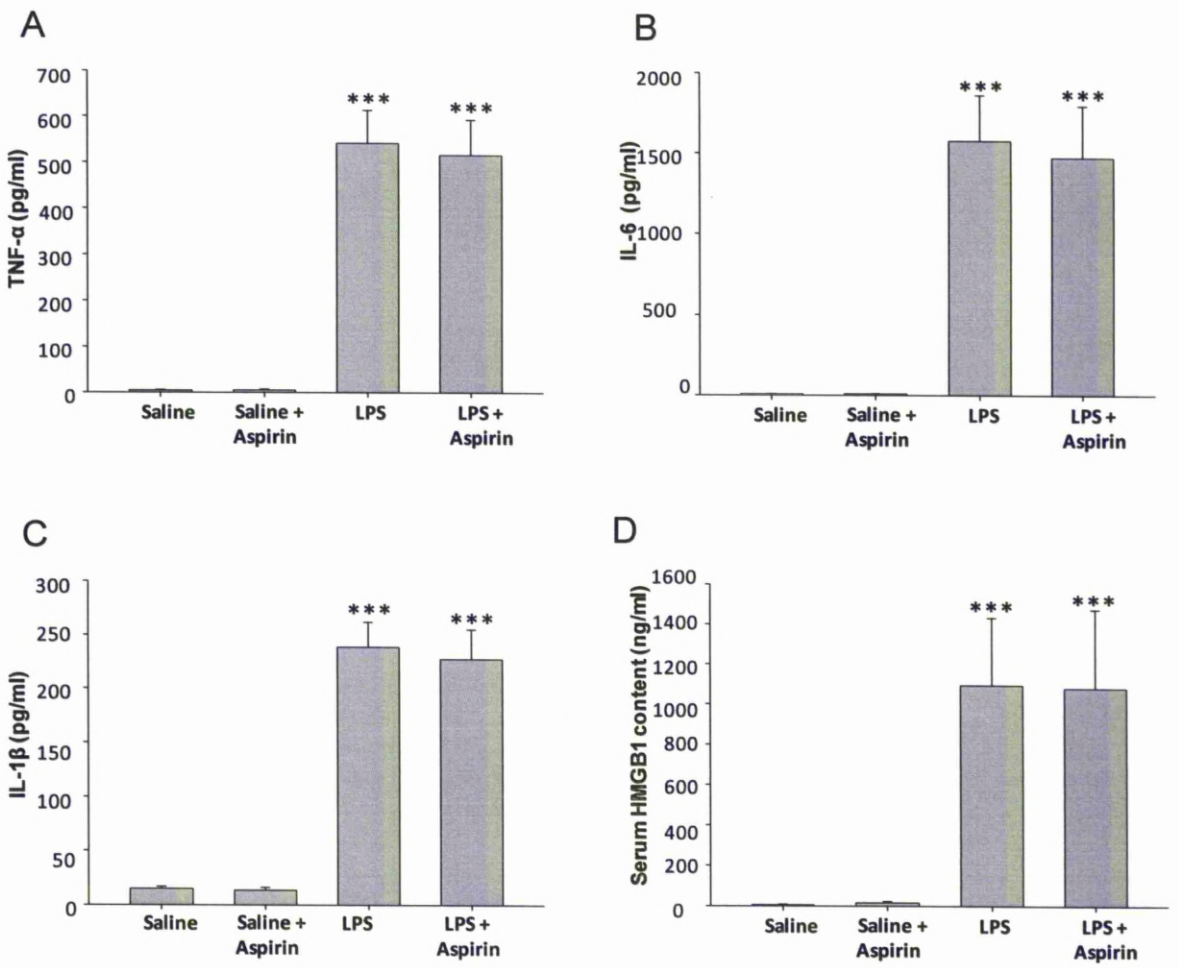

Figure 3.5: Effect of aspirin on serum cytokine levels and HMGB1 during LPS-induced hepatotoxicity in C57BL/6 mice.

C57BL/6 mice were pretreated with or without aspirin (0.06mg/ml) in drinking water for 3 days before administration of LPS (5mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B), IL-1β (C) and serum HMGB1 (D) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. ***$p < 0.001$.

The histological examination confirmed the biochemical findings. There were no findings indicating that aspirin had any effect on the liver. However, it also appeared not to have an effect on the LPS-induced changes, i.e. neutrophil recruitment into the liver and individual hepatocyte death (Table 3.2)

Table 3.2: Histological findings after aspirin and LPS treatment. C57BL/6 mice were pretreated with or without aspirin (0.06mg/ml) in drinking water for 3 days before administration of LPS (5mg/kg). Animals were examined 24hr post LPS treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 5 animals per group.

Treatment	**Score**	**Histological findings**
Saline	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
Saline + Aspirin	0	Diffuse glycogen; NHAIR
LPS	0	Some leukocytes in vessel lumina, scattered neutrophils between hepatic cords, scattered degenerate/necrotic hepatocytes
LPS + Aspirin	0	Diffuse glycogen, several leukocytes in vessel lumina and between hepatic cords, some neutrophils surrounding vessels; occasional degenerate/necrotic hepatocyte

3.3.3 Investigation into the effect of aspirin on GalN-induced hepatotoxicity and inflammation

The effects of aspirin on the reduction of toxicity by modulation of inflammation were investigated in the GalN mouse model of hepatotoxicity and inflammation characterised in Chapter 2. In this study a high dose of aspirin (1mg/ml) was used as no significant effect on the inflammatory response was observed in the LPS model. GalN administered alone caused toxicity as assessed by serum ALT activity (714.15± 58.28U/l) (Figure 3.6A). Aspirin in the drinking water did not have any toxic effect (ALT activity of 81.85±63.55U/l) nor was it capable of reducing the GalN-induced liver injury (ALT activity of 625.05±100.91U/l). Aspirin pretreatment did not alter the hepatic GSH content compared to control (54.02±6.85nmol/mg), nor did GalN alone or in combination with aspirin (58.89±7.19nmol/mg and 59.22±7.20nmol/mg respectively) (Figure 3.6B).

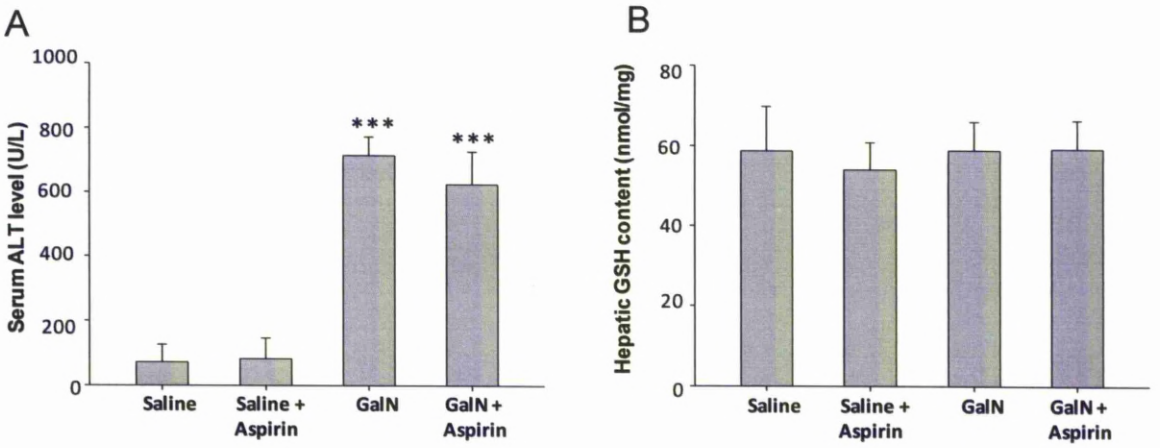

Figure 3.6: The effect of aspirin on GalN-induced hepatotoxicity in male C57BL/6 mice. Serum ALT (A), hepatic GSH content (B) were measured in male C57BL/6 mice pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of GalN (800mg/kg; 24hr). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. ***$p < 0.001$.

Aspirin pretreatment (1mg/ml) did not initiate cytokine release (Figure 3.7), whereas GalN (800mg/kg) caused a significant increase in the serum level of TNF-α at 24hr (90.57±6.45pg/ml) (Figure 3.7A) and a significant increase in serum IL-6 (63.42±8.38pg/ml) (Figure 3.7B) as well as serum IL-1β (70.92±9.08pg/ml) at the same time point (Figure 3.7C). Pretreatment with aspirin (1mg/ml) did not significantly reduce GalN-induced cytokine release, although levels of serum TNF-α (82.72±7.81pg/ml), IL-6 (57.24±6.83pg/ml) and IL-1β (64.50±5.87pg/ml) were each lower than those after GalN treatment alone.

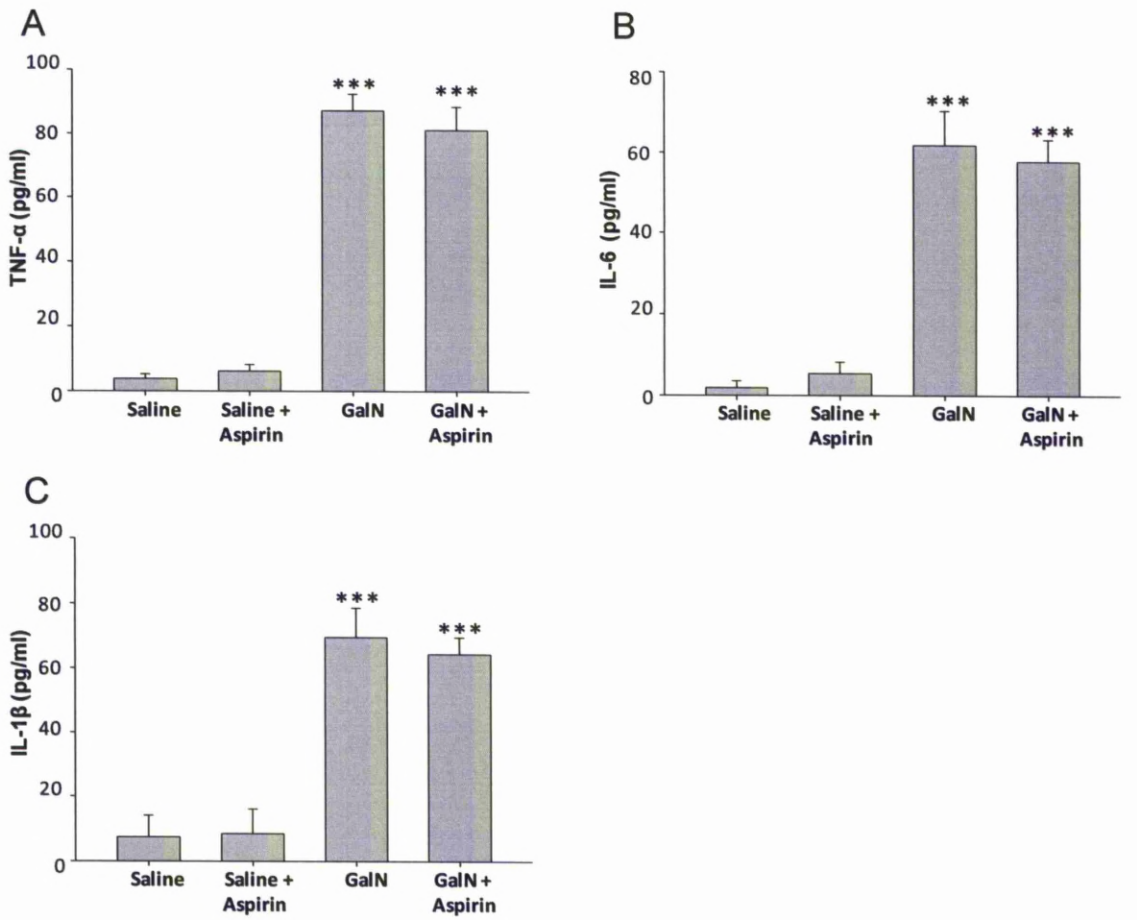

Figure 3.7: Effect of aspirin on serum cytokine levels during GalN-induced hepatotoxicity in C57BL/6 mice.
Male C57BL/6 mice were pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of GalN (800mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. ***$p < 0.001$.

3.3.4 Investigation into the effect of aspirin on APAP-induced hepatotoxicity and metabolism

The effects of aspirin on the reduction of toxicity and possible effects on APAP metabolism were investigated in the APAP mouse model of hepatotoxicity characterised in Chapter 2. This study was repeated with an aspirin dose identical to the previously published dose (0.06mg/ml in drinking water; Imaeda et al., 2009) and a similar time course (10hr) to directly compare results. Aspirin in the drinking water caused no toxicity as assessed by serum ALT activity (24.17±3.88U/l) nor did it reduce APAP hepatotoxicity compared to APAP-treated mice with normal drinking water (3143.15±215.23U/l and 3276.01±305.45U/l respectively) (Figure 3.8A). Aspirin pretreatment did not alter hepatic GSH levels compared to control (47.52±9.66nmol/mg and 47.29±8.17nmol/mg respectively) (Figure 3.8B). At 10hr post dosing the GSH content was lower in APAP-treated mice compared to control mice (40.17±5.56nmol/mg) although this reduction was not statistically significant. Aspirin pretreatment did not cause any difference in GSH recovery after APAP overdose, with levels returning to those of controls (43.47±11.71nmol/mg). To investigate the effect of aspirin on APAP metabolism the hepatic GSH content was measured 1hr after APAP-treatment (530mg/kg). At 1hr, aspirin pretreatment did not alter hepatic GSH levels compared to control (72.70±3.13nmol/mg) whereas APAP caused a significant decrease in GSH levels (15.11±3.61nmol/mg) (Figure 3.9). Aspirin did not affect APAP metabolism as GSH levels were significantly decreased similar to those after APAP treatment alone (17.48±2.07nmol/mg).

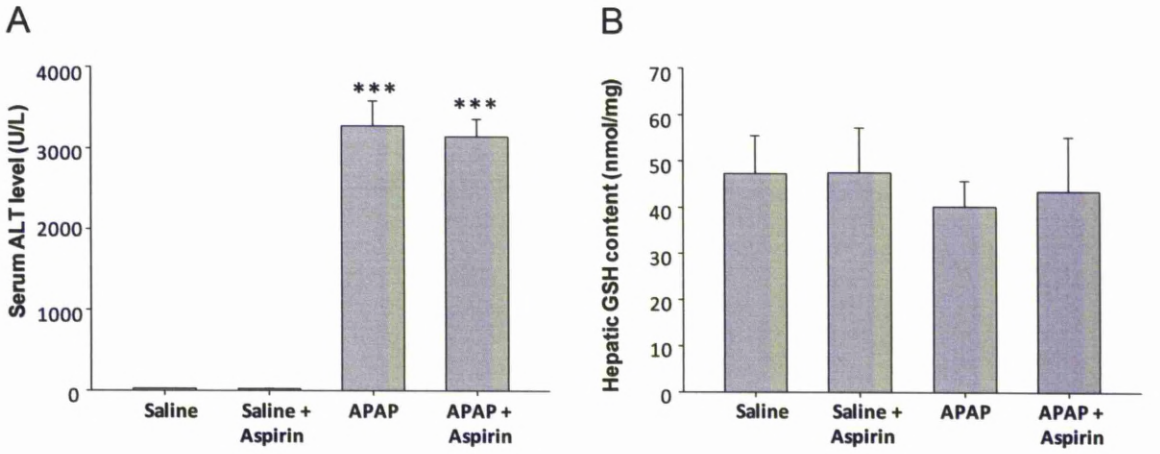

Figure 3.8: The effect of aspirin pretreatment on APAP-induced hepatotoxicity in fasted C57BL/6 mice.

Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice pretreated with or without aspirin (0.06mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 10hr). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. ***$p < 0.001$.

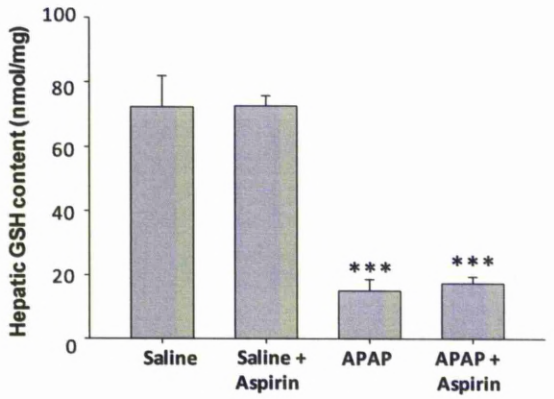

Figure 3.9: The effect of aspirin on bioactivation of APAP.

Hepatic GSH content was evaluated in male C57BL/6 mice were pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 1hr). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to saline control. ***$p < 0.001$.

3.3.5 Investigation into the effect of aspirin on APAP-induced hepatotoxicity and inflammatory infiltration in fasted C57BL/6 mice at 10hr

The effects of aspirin on the reduction of toxicity by modulation of inflammation were investigated in the fasted APAP C57Bl/6 model. At a similar time point to that previously published (10hr) an increasing dose of aspirin (0.06-1mg/ml) was used to assess if any dose dependent modulation of inflammation and potential subsequent effects on APAP-induced hepatotoxicity would occur. Aspirin pretreatment of increasing dose caused no toxicity as measured by serum ALT activities (Figure 3.10A). A significant decrease of APAP-induced toxicity was observed with aspirin concentrations of 0.3 and 0.6mg/ml (3008.90±309.06U/l and 2988.05±103.57U/l respectively) compared to mice treated with APAP alone (530mg/kg; 3390.83±291.42U/l). Aspirin did not alter the hepatic GSH content compared to control animals (44.00±2.27nmol/mg) whereas APAP caused a significant decrease (29.95±2.31nmol/mg) (Figure 3.10B). Aspirin (0.06-0.6mg/ml) did not aid GSH content recovery in APAP-treated groups compared to their respective controls, whereas at 1mg/ml there was an observable difference in GSH content between control and APAP dosed mice but the difference was not significant (44.07±1.78nmol/mg and 31.73±2.85nmol/mg respectively).

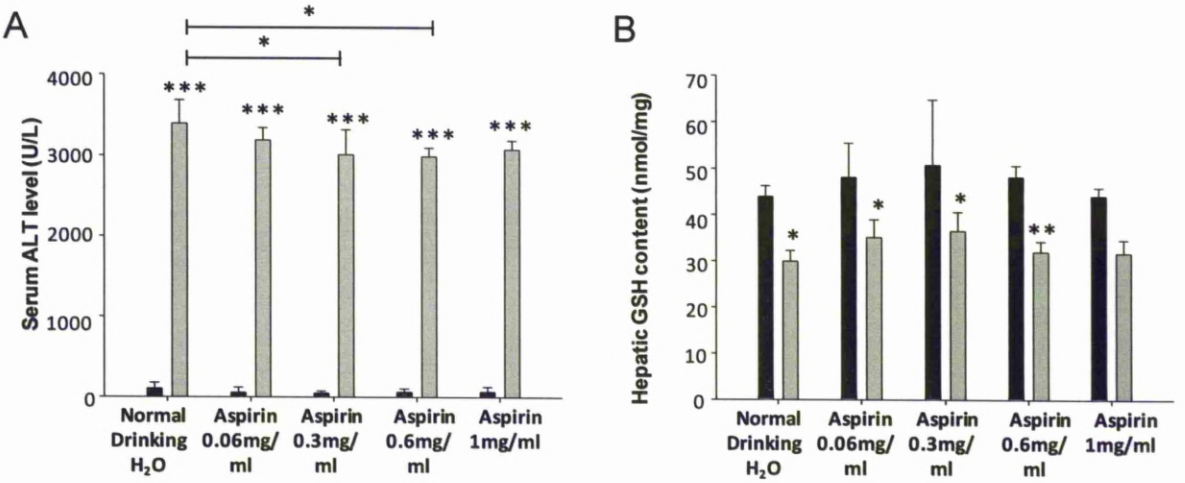

Figure 3.10: The effect of increasing dose of aspirin on APAP-induced hepatotoxicity in fasted C57BL/6 mice at 10hr.
Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice pretreated with or without aspirin (0.06-1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 10hr). (Saline, black; APAP, grey). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Aspirin pretreatment (1mg/ml) did not induce cytokine release into the serum (Figure 3.11). APAP (530mg/kg) caused a significant increase in the serum level of TNF-α at 10hr (223.74±20.28pg/ml) (Figure 3.11A). IL-6 also increased at 10hr post APAP dose (149.63±14.74pg/ml) (Figure 3.11B) as well as levels of IL-1β (48.42±6.49pg/ml) (Figure 3.11C). Pretreatment with aspirin (1mg/ml) significantly reduced levels of APAP-induced TNF-α release after 10hr (183.56±16.51pg/ml) but did not significantly alter levels of IL-6 or IL-1β (123.41±13.36pg/ml and 39.46±4.99pg/ml respectively) despite an observed trend of reduction for both cytokines.

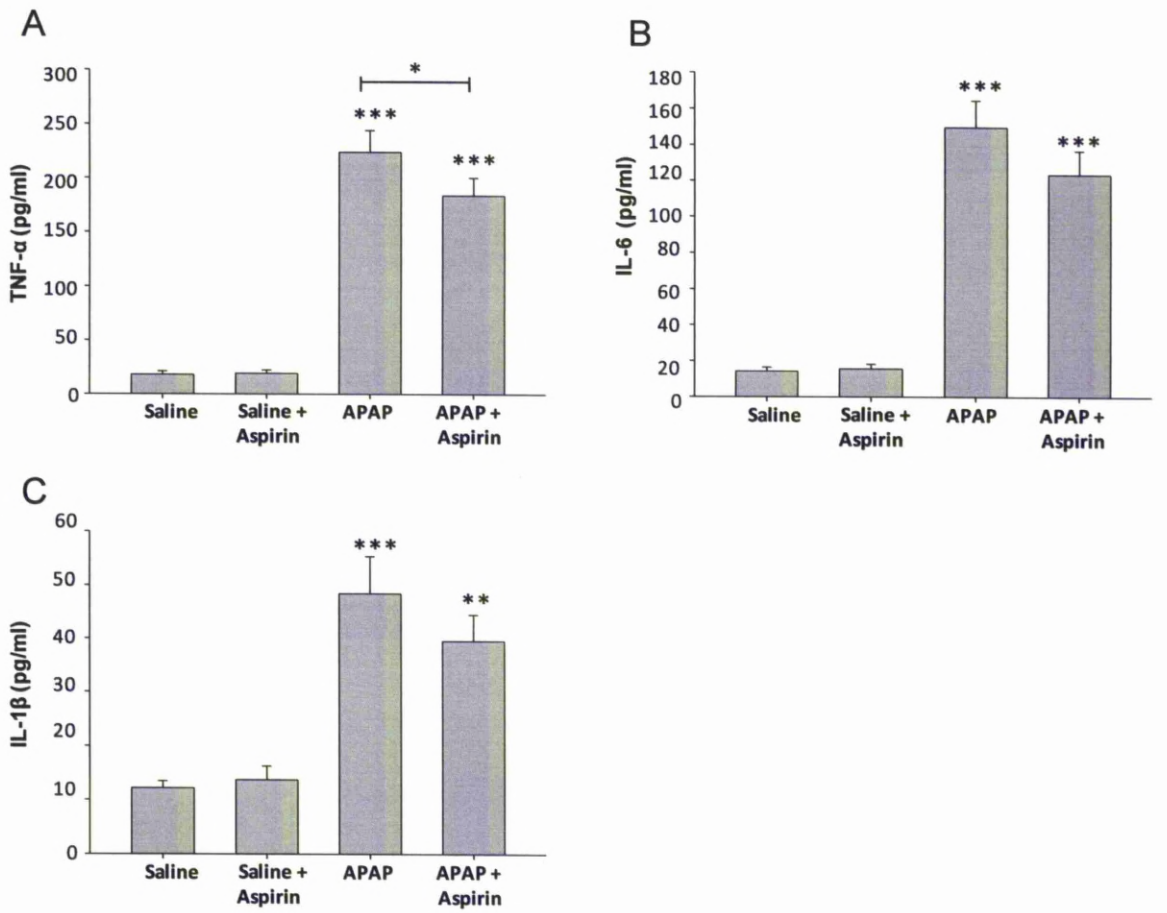

Figure 3.11: Effect of aspirin pretreatment on serum cytokine levels during APAP-induced hepatotoxicity in fasted C57BL/6 mice at 10hr.
Fasted male C57BL/6 mice were pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 10hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

The histological examination confirmed that aspirin does not have an effect on neutrophil recruitment into the liver after APAP-induced injury. Instead the average scores of mice treated with the different doses of aspirin were higher than in mice treated with APAP alone (Table 3.3).

Table 3.3: Histological findings after aspirin and APAP treatment in fasted mice at 10hr. Fasted male C57BL/6 mice were pretreated with or without aspirin (0.6-1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg). Animals were examined 10hr after APAP treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 5 animals per group.

Treatment	**Score [average]**	**Histological findings**
Saline	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
APAP (530mg/kg)	1-2 [1.5]	Affected areas surrounded by rim of glycogen loss and some degree of hydropic degeneration; several neutrophils and individual necrotic cells in some affected areas
Aspirin (0.6mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (0.6mg/kg) + APAP	2-4 [3.3]	Affected areas with extensive hydropic degeneration and necrosis, surrounded by rim of hydropic degeneration and some neutrophils in affected areas; no glycogen
Aspirin (1mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (1mg/kg) + APAP	2-4 [2.6]	Affected areas with extensive hydropic degeneration and necrosis, relatively numerous neutrophils (also as small aggregates and in central veins); some hydropic degeneration of hepatocytes outside affected areas; no glycogen

3.3.6 Investigation into the effect of aspirin on APAP-induced hepatotoxicity and inflammation in fasted C57BL/6 mice at 24hr

The previous study investigating the effects of increasing levels of aspirin on APAP-induced toxicity and modulation of inflammation in fasted C57BL/6 mice was repeated with an extended time course of 24hr to assess if potential effects of aspirin are more pronounced at a later time point. Similar to 10hr, aspirin pretreatment of increasing dose at 24hr post APAP dose (530mg/kg) caused no toxicity as measured by serum ALT activities (Figure 3.12A). Aspirin (0.06-1mg/ml) did not alter the hepatic GSH content compared to control animals (36.80±24.49nmol/mg) (Figure 3.12B). APAP caused a significant decrease in hepatic GSH content at an aspirin concentration of 0.3mg/ml (30.99±12.77nmol/mg) with the other APAP-aspirin treated groups returning to control levels. The histological examination confirms that aspirin does not have an effect on APAP-induced hepatotoxicity and inflammatory cell recruitment at 24hr post treatment (Table 3.4).

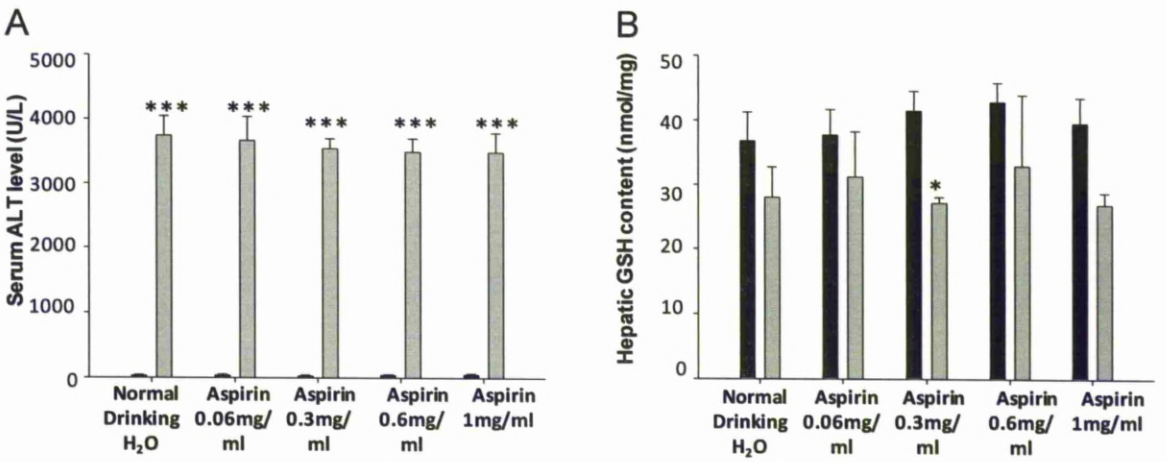

Figure 3.12: The effect of increasing dose of aspirin on APAP-induced hepatotoxicity in fasted C57BL/6 mice at 24hr.
Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice pretreated with or without aspirin (0.06-1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 24hr). (Saline, black; APAP, grey). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. *$p < 0.05$, ***$p < 0.001$.

Aspirin pretreatment (1mg/ml) did not induce cytokine release into the serum (Figure 3.13). APAP (530mg/kg) caused a significant increase in the serum level of TNF-α (247.41±24.58pg/ml; Figure 3.13A), IL-6 (208.51±11.82pg/ml; Figure 3.13B) and IL-1β (45.75±4.76pg/ml Figure 3.13C) at 24hr. Pretreatment with aspirin (1mg/ml) did not significantly alter the serum levels of the three cytokines.

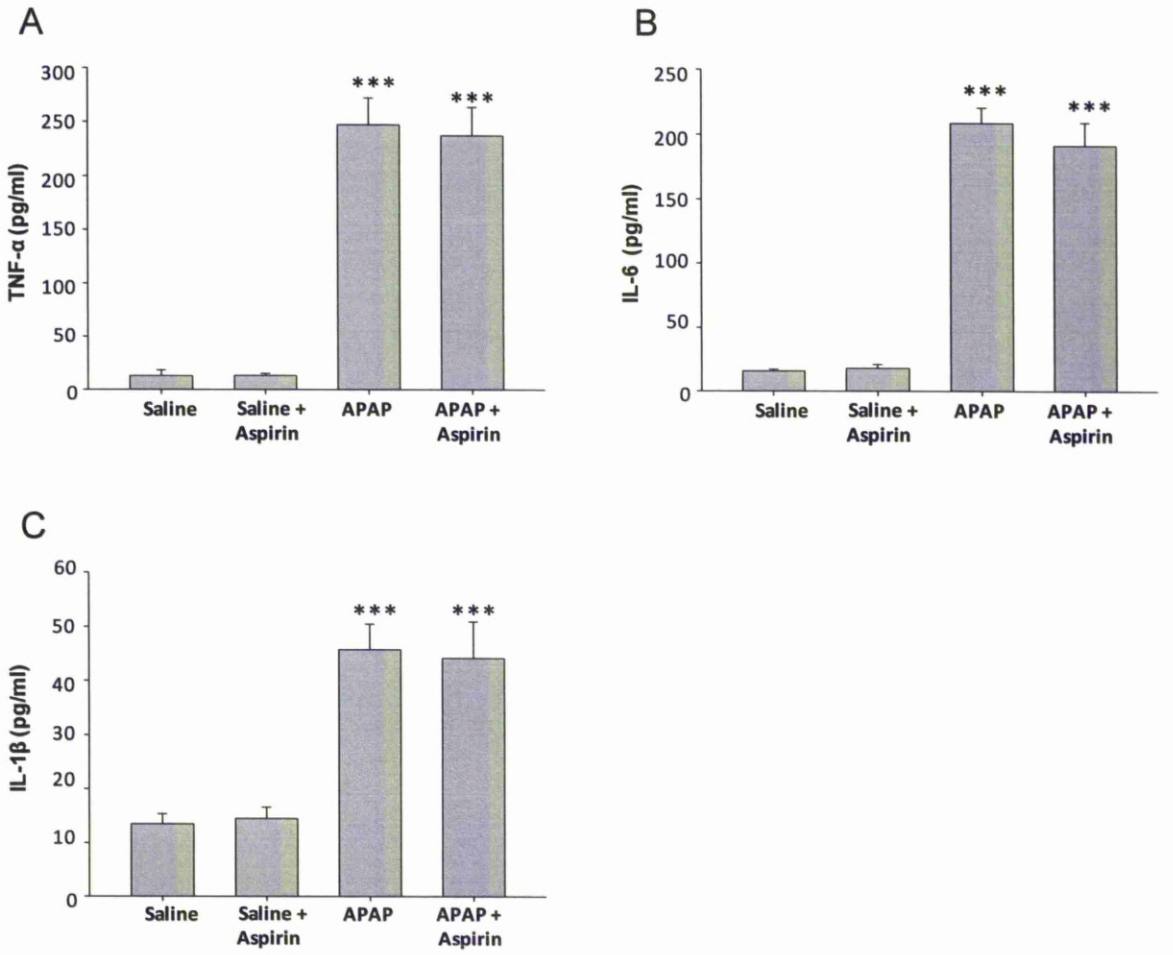

Figure 3.13: Effect of aspirin on serum cytokine levels during APAP-induced hepatotoxicity in fasted C57BL/6 mice at 24hr.
Fasted male C57BL/6 mice were pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table 3.4: Histological findings after aspirin and APAP treatment in fasted mice at 24hr. Fasted male C57BL/6 mice were pretreated with or without aspirin (0.06-1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 24hr). The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 5 animals per group.

Treatment	**Score [average]**	**Histological findings**
Saline	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
APAP (530mg/kg)	2-4 [2.63]	Hydropic degradation in affected areas and surrounding these; some neutrophils in affected areas and central veins; diffuse glycogen outside affected areas
Aspirin (0.06mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (0.06mg/kg) + APAP	0-5 [2.5]	No glycogen; sharply delineated affected areas with hydropic degeneration of intact cells surrounding these and coagulative necrosis (several individual and groups of necrotic cells; occasional apoptotic cells); a few neutrophils in affected areas (also in central veins)
Aspirin (0.3mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (0.3mg/kg) + APAP	2-5 [3.25]	No glycogen; sharply delineated affected areas with hydropic degeneration and coagulative necrosis (numerous individual necrotic and apoptotic cells, at border to unaltered tissue); a few neutrophils
Aspirin (0.6mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (0.6mg/kg) + APAP	2-4 [3.5]	No glycogen; hydropic degeneration of cells surrounding affected areas; numerous necrotic/apoptotic cells in affected areas; relatively numerous neutrophils
Aspirin (1mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (1mg/kg) + APAP	2-5 [3.83]	No glycogen; sharply delineated affected areas with hydropic degeneration and coagulative necrosis (numerous individual necrotic and apoptotic cells, at border to unaltered tissue); some hydropic degeneration surrounding affected areas; some neutrophils in affected areas

3.3.7 Investigation into the effect of aspirin on APAP-induced hepatotoxicity and inflammation in non-fasted C57BL/6 mice at 10hr

The effects of aspirin on the reduction of APAP-induced toxicity by modulation of inflammation were investigated this time in the non-fasted C57Bl/6 model at the 10hr time point to see if potential effects of aspirin on APAP-induced hepatotoxicity are more pronounced in fed animals. Aspirin pretreatment of increasing dose caused no toxicity as measured by serum ALT activities (Figure 3.14A). A significant decrease of APAP-induced toxicity was observed with an aspirin concentration of 0.6mg/ml (1654.02±224.49U/l) compared to mice treated with APAP alone (530mg/kg; 2031.77±181.27U/l). Aspirin did not alter the hepatic GSH content compared to control animals (72.93±8.42nmol/mg) whereas APAP caused a significant decrease (47.00±4.05nmol/mg) (Figure 3.14B). No significant reduction in GSH content was observed in APAP-treated groups of higher concentrations of aspirin (0.6-1mg/ml) (50.12±8.57nmol/mg and 50.11±9.63nmol/mg respectively). The histological examination confirms that aspirin pre-treatment does not have an effect on APAP-induced hepatotoxic changes and neutrophil recruitment into the liver 10hr post treatment (Table 3.5).

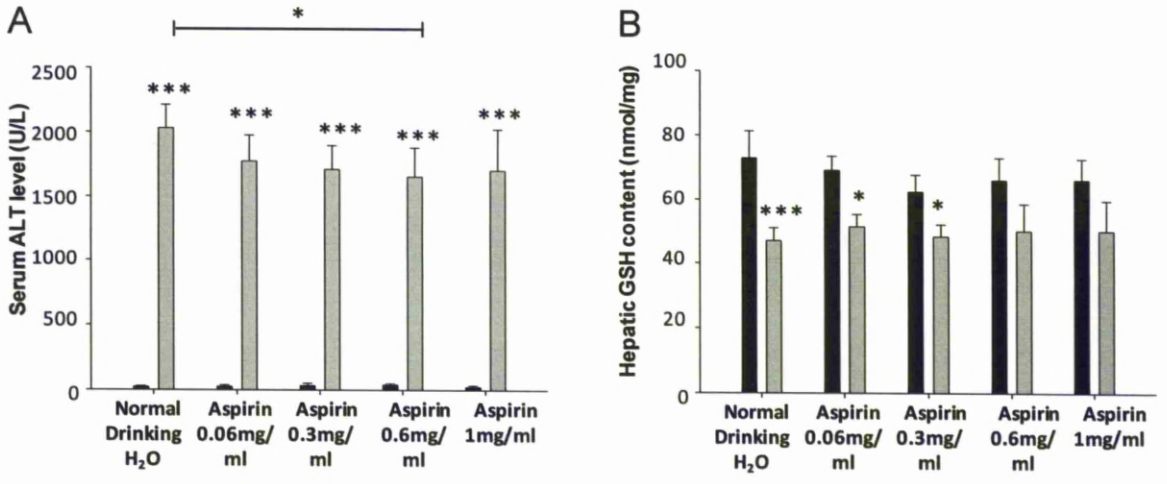

Figure 3.14: The effect of increasing dose of aspirin on APAP-induced hepatotoxicity in non-fasted C57BL/6 mice at 10hr.
Serum ALT (A) and hepatic GSH content (B) were measured in non-fasted male C57BL/6 mice pretreated with or without aspirin (0.06-1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 10hr). (Saline, black; APAP, grey). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. *$p < 0.05$, ***$p < 0.001$.

Aspirin pretreatment (1mg/ml) did not induce cytokine release into the serum (Figure 3.15). APAP (530mg/kg) caused a significant increase in the serum level of TNF-α (78.31±6.54pg/ml; Figure 3.15A), IL-6 (78.85±7.09pg/ml; Figure 3.15B) and IL-1β (24.54±4.06pg/ml Figure 3.15C) at 10hr. Pretreatment with aspirin (1mg/ml) significantly reduced APAP-induced serum TNF-α levels after 10hr (65.91±3.26pg/ml) but did not significantly alter serum levels of IL-6 or IL-1β (69.97±4.80pg/ml and 18.26±2.98pg/ml respectively) despite an observed trend of reduction for both cytokines.

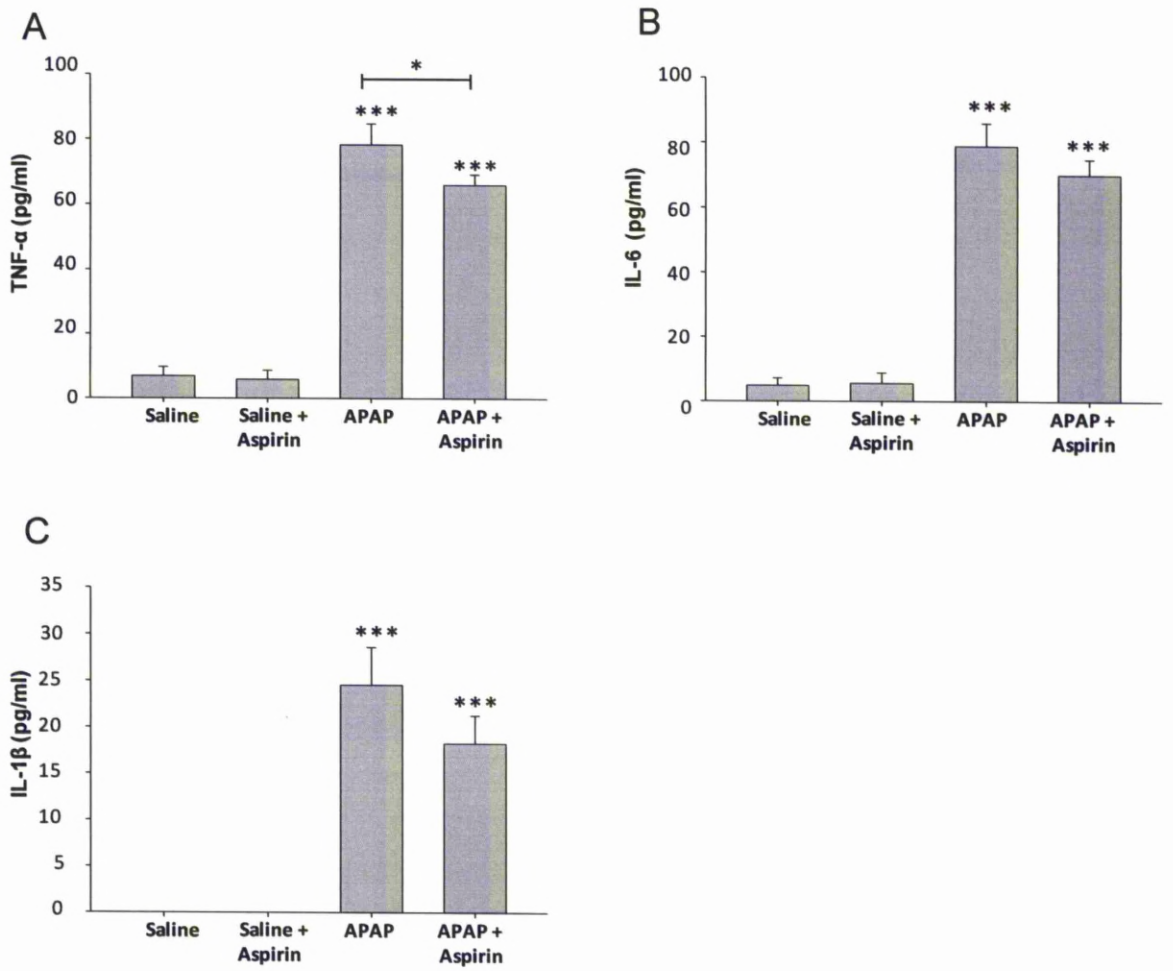

Figure 3.15: Effect of aspirin on serum cytokine levels during APAP-induced hepatotoxicity in non-fasted C57BL/6 mice at 10hr.
Non-fasted male C57BL/6 mice were pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 10hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. *$p < 0.05$, ***$p < 0.001$.

Table 3.5: Histological findings after aspirin and APAP treatment in non-fasted mice at 10hr. Male C57BL/6 mice were pretreated with or without aspirin (1mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 10hr). The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 4 animals per group.

Treatment	**Score [average]**	**Histological findings**
Aspirin (1mg/kg) + Saline	0	No glycogen; no histological abnormality is recognised (NHAIR)
Aspirin (1mg/kg) + APAP	0-5	No glycogen; hydropic degeneration;surrounding areas of cell loss; occasional apoptotic/necrotic hepatocytes; a few neutrophils in affected areas (border of cell loss)

3.3.8 Investigation into the effect of aspirin on APAP-induced hepatotoxicity and inflammation in non-fasted C57BL/6 mice at 24hr

The previous study on the effects of increasing levels of aspirin on APAP-induced toxicity and modulation of inflammation in non-fasted C57Bl/6 mice was repeated with an extended time course of 24hr. Aspirin pretreatment of increasing dose (0.06-0.6mg/ml) at 24hr post APAP dose (530mg/kg) caused no toxicity as measured by serum ALT activities (Figure 3.16A). Aspirin (0.06-0.06mg/ml) did not alter the hepatic GSH content compared to control animals (71.42±3.42nmol/mg) (Figure 3.16B). The histological examination confirms that aspirin treatment does not have a distinct effect on APAP-induced hepatotoxic changes and the degree of neutrophil recruitment into the liver 24hr post treatment (Table 3.6).

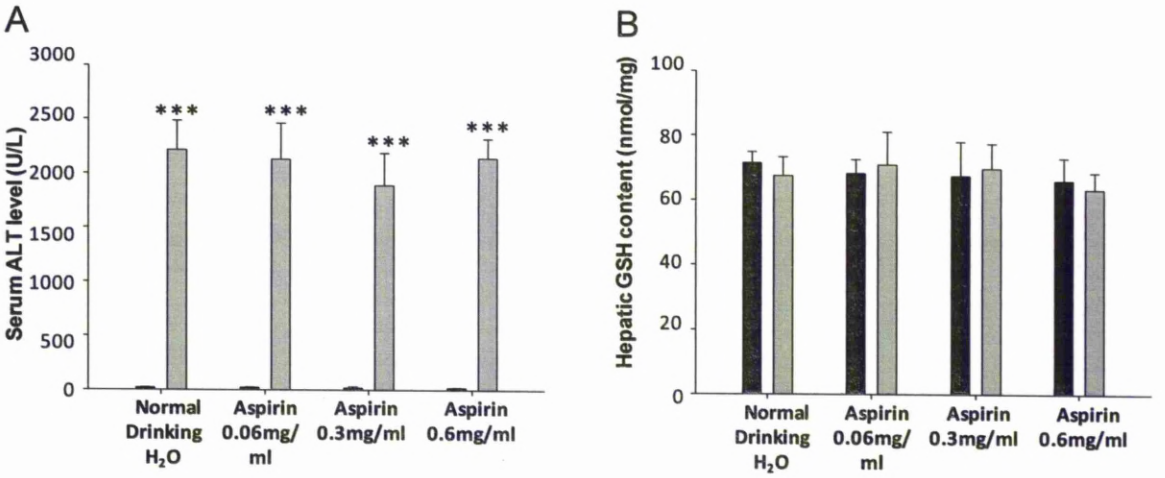

Figure 3.16: The effect of increasing dose of aspirin on APAP-induced hepatotoxicity in non-fasted C57BL/6 mice at 24hr.
Serum ALT (A) and hepatic GSH content (B) were measured in non-fasted male C57BL/6 mice pretreated with or without aspirin (0.06-0.6mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 24hr). (Saline, black; APAP, grey). Data is given as mean ± SD of 5 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. ***$p < 0.001$.

Aspirin pretreatment (0.6mg/ml) did not induce cytokine release into the serum (Figure 3.17). APAP (530mg/kg) caused a significant increase in the serum level of TNF-α (86.16±7.22pg/ml; Figure 3.17A), IL-6 (122.89±12.21pg/ml; Figure 3.17B) and IL-1β (18.56±6.55pg/ml Figure 3.17C) at 24hr post dose. Pretreatment with aspirin (0.6mg/ml) did not significantly alter the levels of the three cytokines after APAP treatment.

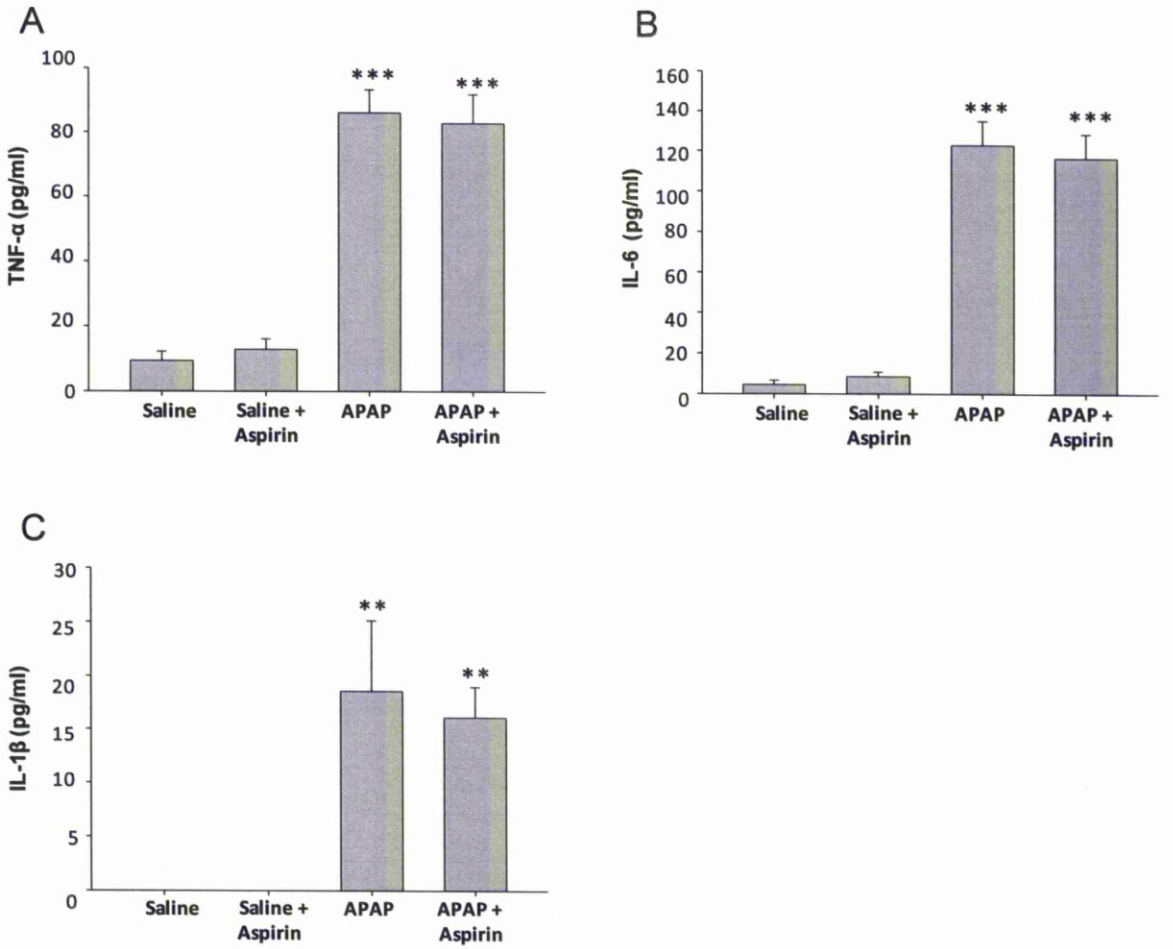

Figure 3.17: Effect of aspirin on serum cytokine levels during APAP-induced hepatotoxicity in non-fasted C57BL/6 mice at 24hr.

Non-fasted male C57BL/6 mice were pretreated with or without aspirin (0.6mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined. Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without aspirin. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3.6: Histological findings after aspirin and APAP treatment in non-fasted mice at 24hr. Male C57BL/6 mice were pretreated with or without aspirin (0.06-0.6mg/ml) in drinking water for 3 days before administration of APAP (530mg/kg; 24hr). The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 4 animals per group.

Treatment	**Score [average]**	**Histological findings**
Saline	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
APAP (530mg/kg)	0-4 [2.25]	No glycogen; hydropic degeneration of remaining hepatocytes in affected areas; some neutrophils in blood-filled centrilobular areas and central veins
Aspirin (0.06mg/kg) + Saline	0	Diffuse glycogen, often low amount; NHAIR
Aspirin (0.06mg/kg) + APAP	0-4 [2.43]	In affected areas often inner area of cell loss with haemorrhage, surrounded by coagulative necrosis of hepatocytes and rim of glycogen loss
Aspirin (0.3mg/kg) + Saline	0	Variable amount of hepatocytes with glycogen; NHAIR
Aspirin (0.3mg/kg) + APAP	0-4 [1.63]	Centrolobular cell loss and coagulative necrosis; no glycogen; some neutrophils in affected areas
Aspirin (0.6mg/kg) + Saline	0	Diffuse glycogen; NHAIR
Aspirin (0.6mg/kg) + APAP	0-4 [2]	Area of cell loss surrounded by cells with hydropic degeneration; no glycogen or broad centrilobular rim of glycogen loss; a few neutrophils in affected areas

3.3.9 Comparison of the effects of aspirin on APAP-induced hepatotoxicity and inflammation in fasted and non-fasted C57BL/6 mice

Table 3.7 shows a comparison of the effects of high dose aspirin on APAP-induced hepatotoxicity and inflammation in fasted and non-fasted C57BL/6 mice at 10hr and 24hr post APAP treatment. The table illustrates that APAP-induced hepatotoxicity as assessed by serum ALT activity was significantly greater in fasted C57BL/6 mice compared to fed mice (3390.83±291.42U/l and 2031.77±181.27U/l respectively) at 10hr post APAP (530mg/kg). The trend was also similar at 24hr post APAP with significantly higher ALTs in fasted mice (3747.27±301.90U/l) than in fed mice (2214±266.82U/l). Pretreatment with aspirin reduced serum ALT activity by 16% in fed mice compared to 9.3% in fasted mice at 10hr post APAP dose. At 24hr post APAP dose, aspirin reduced ALT activity by a greater extent in fasted mice than in fed mice (6.9% and 3.3% respectively) although the extent of this reduction for both fasted and fed mice at 24hr was less than that observed at 10hr and the overall effect of aspirin on APAP toxicity were not significant. Pretreatment with aspirin reduced serum protein levels of TNF-α by 17.9% in fasted mice with a similar trend in fed mice (15.8%) at 10hr post APAP dose. At 24hr post APAP dose, aspirin reduced serum TNF-α in fasted mice by 4.1% and fed mice by 3.8%. Similar to TNF-α, aspirin pretreatment reduced levels of serum IL-6 by 17.5% in fasted mice compared to 11.3% in fed mice at 10hr post APAP dose and the same trend was observed 24hr post APAP. In fed mice the serum protein levels of IL-1β were reduced to a greater extent compared to fasted mice at both 10hr (25% fed and 18.5% fasted) and 24hr (13.7% fed and 3.5% fasted). The results show the trend of a greater level of reduction of serum cytokine levels with aspirin pretreatment at the 10hr time point compared to 24hr post APAP dose. While it can be seen that the levels of toxicity and cytokine release are greater in fasted than in fed mice, the effect of aspirin on reducing toxicity and inflammation in both fasted and fed animals was not significant.

Table 3.7: Summary and comparison of the effect of aspirin on fasted and non-fasted male C57BL/6 mice (APAP 530mg/kg; 10hr and 24hr) Statistical significance was assigned for non-fasted relative to fasted C57BL/6 mice. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

	Fed				Fasted			
	Saline	**Saline + Aspirin**	**APAP**	**APAP + Aspirin**	**Saline**	**Saline + Aspirin**	**APAP**	**APAP + Aspirin**
10hr after dose								
Age (weeks)	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6
Starting body weight (g)	20.33 (0.89)	19.97 (0.93)*	20.33 (1.00)	19.74 (0.72)	18.69 (1.78)	18.26 (0.93)	18.98 (0.52)	18.63 (0.66)
Serum ALT (U/l)	23.71 (5.55)	24.53 (9.99)	2031.77 (181.27)***	1704.25 (320.38)***	105.50 (73.24)	67.16 (62.62)	3390.83 (291.42)	3075.47 (107.85)
Hepatic GSH content (nmol/g)	72.93 (8.42)**	66.17 (6.57)**	47.00 (4.05)***	50.11 (9.63)**	44.00 (2.27)	44.07 (1.78)	29.95 (2.31)	31.73 (2.85)
TNF-α (pg/ml)	6.79 (2.85)*	5.77 (2.82)**	78.31 (6.54)**	65.91 (3.26)**	17.83 (3.40)	19.13 (3.05)	223.74 (20.28)	183.56 (16.51)
IL-6 (pg/ml)	4.92 (2.26)**	5.60 (3.17)*	78.85 (7.09)**	69.97 (4.80)**	14.13 (2.19)	15.60 (2.41)	149.63 (14.74)	123.41 (13.36)
IL-1β (pg/ml)	-	-	24.54 (4.06)**	18.26 (2.98)**	12.19 (1.29)	13.73 (2.54)	48.42 (6.94)	39.46 (4.99)
24hr after dose								
Age (weeks)	4-6	4-6	4-6	4-6	4-6	4-6	4-6	4-6
Starting body weight (g)	21.66 (1.09)***	21.07 (0.79)**	21.06 (0.77)***	19.92 (0.82)**	17.15 (0.39)	17.72 (1.33)	17.77 (0.90)	17.47 (0.61)
Serum ALT (U/l)	19.82 (3.32)	20.02 (2.42)*	2214.92 (266.82)***	2141.11 (175.70)***	28.23 (12.01)	51.39 (12.73)	3747.27 (301.90)	3485.71 (301.46)
Hepatic GSH content (nmol/g)	71.42 (3.42)***	66.05 (6.92)***	67.48 (5.74)***	63.31 (5.19)***	36.80 (4.49)	39.57 (3.99)	28.02 (4.71)	26.88 (1.84)
TNF-α (pg/ml)	9.38 (2.81)	12.87 (3.24)	86.16 (7.22)**	82.83 (9.12)**	12.64 (5.59)	13.06 (1.98)	247.41 (24.58)	237.30 (26.36)
IL-6 (pg/ml)	4.44 (2.20)**	8.44 (2.33)*	122.89 (12.21)**	116.44 (12.15)**	15.59 (1.53)	17.78 (3.14)	208.51 (11.82)	191.44 (17.53)
IL-1β (pg/ml)	-	-	18.65 (6.55)**	16.09 (2.87)**	13.53 (1.84)	14.51 (2.12)	45.75 (4.76)	44.13 (6.79)

3.4 DISCUSSION

There has recently been controversy reported regarding the role of the innate immune system in the overall outcome of drug-induced hepatotoxicity with conflicting literature reports concerning progression and exacerbation of toxicity (Liu et al., 2006; Masson et al., 2008; Williams et al., 2011). Questions about the effects that the solvent DMSO has on APAP-induced liver injury have been raised. Therefore, the aim of one of the studies described within this chapter was to determine if DMSO can be the main cause of hepatic immune cell infiltration during APAP-induced hepatotoxicity through the use of the APAP model of toxicity characterised in Chapter 2. In order to test whether APAP or DMSO is responsible for immune cell infiltration, high doses of APAP were compared to DMSO treatments. The second objective of this chapter was to further investigate the contribution of the immune system in DILI by using aspirin, as it was reported to be a pharmacological inhibitor of the Nalp3 inflammasome in mice (Imaeda et al., 2009).

The use of DMSO to facilitate the dissolution of APAP has been implicated as a major factor in the pathogenic role of NK and in particular, NKT cells in APAP-induced liver injury (Masson et al., 2008). DMSO was implicated as the main cause of hepatic immune cell infiltration with increased numbers of cytotoxic NKT cells in the liver, both in the presence of APAP or alone. It was shown that in addition to affecting the role of NK and NKT cells in APAP-induced hepatotoxicity, the presence of DMSO also decreased the severity of liver injury at equivalent doses of APAP. This effect was thought to be likely due to the inhibition of APAP bioactivation by DMSO (Masson et al., 2008).

A comparison of APAP and DMSO treatments in mice was conducted in order to determine if the use of the solvent DMSO is the main factor involved in immune cell infiltration rather than from APAP alone. As it has been well documented that high levels of DMSO can have an inhibitory effect on CYP450 metabolism and toxicity (Park et al., 1988; Lind et al., 2000; Dunphy et al., 2007) and that DMSO has anti-oxidant activity (Yamasaki et al., 1988; Dunphy et al., 2007), the effect of DMSO on APAP toxicity was also assessed. At 24hr, mice that were administered DMSO alone (1mg/ml in saline) showed no toxicity as assessed by serum ALT levels and histology, whereas mice that were treated with a dose of APAP shown

to cause liver toxicity (530mg/kg) showed a significantly higher level of ALT activity (Figure 3.1A). DMSO, when administered simultaneously with APAP lead to complete abrogation of toxicity, with ALT levels similar to those after DMSO alone, however, when DMSO was dosed 2hr after APAP, APAP hepatotoxicity was observed. This data is in concordance with previous work (Masson et al., 2008) which suggested that high levels of DMSO could inhibit APAP bioactivation when given at the same time, with toxicity being observed if DMSO treatment was delayed by 2hr, giving time for APAP to become metabolised into its reactive metabolite and cause cell damage. At the equivalent time point (24hr) however, the levels of hepatic GSH content in each dose group were similar to control levels. When DMSO was administered 2hr after APAP, despite ALT levels being high, the GSH content was similar to levels when DMSO was given simultaneously (Figure 3.1B). GSH levels may have been returning to normal after 24hr as a result of liver regeneration and repair. Depletion would have occurred at an earlier time point as a result of oxidative stress caused by APAP, but the cellular levels of GSH would be replenished 24hr after the initial toxic insult. As it was observed that a high level of DMSO (1ml/kg) abrogated APAP-induced hepatotoxicity, a DMSO dose response was used in order to determine what levels could cause this inhibition (Figure 3.2). To assess whether ALT inhibition was also associated with a reduction of biochemical oxidative stress, the level of hepatic GSH content was measured 5hr after APAP exposure, so GSH levels would not have returned to normal. It was seen by serum ALT activity that toxicity was significantly reduced compared to APAP alone when DMSO levels were greater than 0.1ml/kg (Figure 3.2A). At the maximum level of DMSO used (1ml/kg), toxicity was completely abrogated at 5hrs suggesting that bioactivation of APAP was inhibited in the presence of DMSO. GSH levels were significantly reduced in all DMSO dose groups compared to DMSO alone (Figure 3.2B), however, levels increased in a dose dependent manner compared to APAP alone, most notably at levels greater than 0.1ml/kg DMSO. This was in parallel to the reduction in ALT levels at equivalent doses suggesting that some bioactivation was occurring. The time dependent effect of DMSO (1ml/kg) administration on bioactivation and toxicity was also explored (Figure 3.3). As seen with the previously described study, when DMSO was administered concurrently or 1hr after APAP, toxicity was abrogated as shown by the level of ALT activity (Figure 3.3A). The levels of ALT were similar to those of the control groups and significantly lower than with APAP alone. Administration of DMSO 2hr and 4hr after APAP lead to a significant increase in ALT activity compared to control, however, there was still a significant decrease with both time points compared to APAP alone although levels were above the threshold of toxicity. The

levels of hepatic GSH content were significantly reduced in all APAP and DMSO treated groups compared to control, however when DMSO was administered concurrently and 1hr after APAP, the GSH level was significantly higher than after APAP treatment alone suggesting some inhibition of bioactivation (Figure 3.3B). The reduction of bioactivation and toxicity seen within these studies can be attributed to the high levels of DMSO used. The observation that APAP bioactivation is not completely inhibited by DMSO could be accounted for by the fact that APAP is metabolised by multiple CYP450 enzymes. APAP is primarily bioactivated by CYP2E1 and also CYP3A4 and CYP1A2 (Dahlin et al., 1984). A study investigating the effect of organic solvents on CYP enzymes have shown that DMSO can inhibit CYP2E1 and partially CYP3A4 (Easterbrook et al., 2001) which could result in some APAP being bioactivated by any remaining uninhibited CYP3A4 or CYP1A2 enzymes. The histological examination confirmed the biochemical findings, as concurrent administration of DMSO with APAP showed no histological abnormality normally associated with APAP treatment, however, cell loss was evident when DMSO was administered 2hr post APAP treatment.

It was hypothesised that aspirin is capable of inhibiting the Nalp3 inflammasome and it was reported that pretreatment with aspirin in drinking water reduced APAP-induced liver injury (Imaeda et al., 2009). It was concluded that aspirin protected due to inhibition of cytokine transcriptional activation (i.e. IL-1β, IL-18, TNF-α and IFN-γ) and also inhibition of Nalp3, which processes the pro-form of IL-1β and IL-18 (Imaeda et al., 2009). The assumption was that the reduced cytokine formation attenuates liver injury through reduced inflammatory cell activation and recruitment. In this chapter a study was undertaken to repeat the published one and with the aspirin dose previously published (0.06mg/ml in drinking water) as well as with higher doses (0.3mg/ml, 0.6mg/ml and 1mg/ml) in the three animal models established in Chapter 2. The aims of these studies were to see if aspirin could act as a pharmacological inhibitor of the Nalp3 inflammasome and subsequently, reduce inflammation and toxicity in our LPS, GalN and APAP animal models.

The effect of LPS, GalN and APAP on toxicity, metabolism and inflammation were investigated in Chapter 2, therefore, the effects of a pharmacological agent used to modulate these factors could be evaluated. The LPS model of hepatotoxicity and inflammation was first utilised to examine the effect of aspirin on inflammation as LPS produces a substantial

inflammatory response. Using the dose previously published, aspirin in the drinking water of mice caused no liver injury as assessed by serum ALT activity and histology, nor did LPS on its own over a 24hr period (Figure 3.4A), which is in concordance with the findings in Chapter 2. Aspirin pretreatment and LPS alone did not alter hepatic GSH content (Figure 3.4B) or serum cytokine levels (Figure 3.5). Aspirin caused a trend of decreasing LPS induced cytokine release, but the reduction was not statistically significant. The levels of each cytokine produced after administration of LPS were consistent with the levels observed in Chapter 2. Pretreatment of aspirin in the GalN mouse model showed a slight decrease in serum ALT activity compared to GalN alone but the reduction was not significant (Figure 3.6A). In a similar finding to the LPS model, there was a trend of decreasing serum cytokine levels in aspirin pretreated animals with GalN compared to GalN alone but the decrease was again not significant (Figure 3.7). These results with GalN were observed using a much higher dose of aspirin than previously published (1mg/ml; Imaeda et al., 2009). These findings suggest that aspirin was ineffective in our LPS and GalN models at reducing the level of toxicity and inflammation. The latter was also confirmed by histology, since there was no obvious difference in the degree of inflammatory cell recruitment into the liver.

Using the same pretreatment regimen as described by Imaeda et al. (2009) no significant effect of aspirin on APAP-induced hepatotoxicity was observed after 10hr when compared to APAP alone in the APAP C57BL/6 mouse model (Figure 3.8A). APAP metabolism was shown to be unaffected by aspirin pretreatment in our investigation as hepatic GSH levels were significantly reduced from control in APAP with aspirin treated groups, comparable to the level of reduction seen with APAP alone (Figure 3.9), although this was not assessed in the previous study (Imaeda et al., 2009). The APAP-aspirin study was expanded to see if higher doses of aspirin, different time points of APAP exposure and fasting had any effect on the overall effectiveness of aspirin as a protector of toxicity and inflammation. Mice were given a dose range of aspirin (0.06-1mg/ml) prior to APAP administration and examined 10hr or 24hr and had been either fasted or fed. Aspirin pretreatment showed a slight dose-dependent trend to reduce toxicity and cytokine release but the overall effect was not statistically significant. This was further confirmed by the histological examination, where there was no distinct effect on APAP-induced liver changes or inflammatory cell recruitment into the liver. Increasing the time course of APAP-exposure from 10hr to 24hr also did not alter the overall effect of aspirin on toxicity and inflammation. In Chapter 2 it was observed

that at the later time point of 24hr, the extent of toxicity and inflammation in the APAP model was greater than at preceding time points. The resulting hypothesis was that at this later time point, aspirin may have more of an impact on reducing toxicity and inflammation. If the overall protective effect of aspirin was negligible, it might not have been detectable in a model with a lesser toxicological and inflammatory response. A similar hypothesis was formed regarding whether mice were fasted or fed, as the findings from Chapter 2 showed that fasting of APAP-treated mice increased the level of toxicity and cytokine release compared to fed animals, and therefore the effect of aspirin may be detectable in the fasted model. However, the results of the present study showed no significant effect of aspirin on APAP-induced toxicity and inflammation.

These data further support the concept that aspirin pretreatment has no significant effect on the overall extent of experimental APAP hepatotoxicity. These results were also in concordance with data recently published by Williams et al. (2011) who looked at the genetic elimination of the Nalp3 inflammasome as well as pharmacological inhibition of Nalp3 with aspirin in APAP-treated mice. Their data demonstrated that no significant alteration of APAP-induced liver injury occurred in Nalp3 inflammasome knockout mice or wild-type mice treated with aspirin. However the results presented in this Chapter and by Williams et al. (2011), are not consistent with the results and conclusions presented by Imaeda et al. (2009). The only differences in the methods used in this chapter and that of Imaeda were that Imaeda's group used a slightly smaller dose of APAP (500mg/kg) as opposed to 530mg/kg, and that they euthanized their animals at 12hr post APAP treatment, whereas the time points explored in this chapter were 10hr and 24hr post APAP dose. The same dose of aspirin by Imaeda (0.06mg/ml) was used and even increased, but with no bearing on the overall outcome. There are no obvious explanations for the discrepancies in these results, although an important aspect of our study was to show that the use of aspirin as a pharmacological modulator of the inflammatory response in our model did not alter the bioactivation of APAP, something which Imaeda's group did not confirm.

In summary the data presented in this chapter suggest that the effect of DMSO on exacerbation of APAP-toxicity by recruitment of inflammatory cells such as NK/NKT cells is minimal as the inhibitory effect of DMSO on the enzymatic bioactivation of APAP and subsequent reduction of hepatotoxicity is more prominent when DMSO is administered

within 1hr of APAP. The use of DMSO to facilitate the dissolution of compounds in studies involving the hepatic immune system must therefore be carefully considered. The data also strongly supports recent literature evidence and the conclusion that the activation of the Nalp3 inflammasome and subsequent involvement of neutrophils has no relevant impact on APAP hepatotoxicity and therefore may not be a viable therapeutic target to treat APAP overdose. Caution must be exercised in translating this concept to man with respect to aspirin acting as a hepatoprotective agent against APAP-induced liver injury.

CHAPTER FOUR

INVESTIGATION OF THE EFFECT OF ANTI-HMGB1 ANTIBODY AND ETHYL PYRUVATE ON DRUG-INDUCED HEPATOTOXICITY AND LPS-MEDIATED INFLAMMATION

CONTENTS

4.1 INTRODUCTION

The innate immune response has been shown to play an important role in the exacerbation of liver injury during APAP hepatotoxicity downstream of drug metabolism (Ju et al., 2002; Liu et al., 2004; Liu et al., 2006). The contribution to the severity and progression of APAP-induced liver injury is understood to be mediated by recruitment of inflammatory cells into the liver and production of proinflammatory cytokines (Blazka et al., 1995; Scaffidi et al., 2002; Laskin et al., 2003; Liu et al., 2004) which are released from macrophages, neutrophils and other cells of the innate immune system. Chapter 3 focused on the modulation of inflammatory cell recruitment to the liver after initial hepatocyte damage. Along with proinflammatory cytokines such as TNF-α, IL-6, IL-1β, a variety of molecules released from necrotic cells were identified that can induce further cytokine formation. These molecules, collectively termed damage-associated molecular patterns (DAMPs) include DNA fragments, heat shock proteins and HMGB1 (Bianchi et al., 2007).

HMGB1 is a late mediator of lethal systemic inflammation in animal models of cytokine-mediated disease initiated by LPS (Wang et al., 1999). It is passively released by necrotic cells and secreted by activated immune cells but not by cells dying via apoptosis. Following release it acts as a potent inflammatory mediator that has been shown to link the necrotic cell death of hepatocytes to the inflammatory response (Scaffidi et al., 2002) by targeting TLR (Toll-like receptor) and RAGE (Receptor for Advanced Glycation End products) (Kokkola et al., 2005; Yu et al., 2006). HMGB1 is actively released by macrophages after a delay of 12-18hr during endotoxaemia (Wang et al., 1999). Antibodies that neutralise HMGB1 have been shown to protect against the lethal effects of LPS-induced endotoxaemia in mice when administered 2hr after the onset of endotoxaemia. The proinflammatory activity of HMGB1 stimulates the release of TNF-α, IL-1β and other inflammatory cytokines from macrophages and mediates lethality of mice (Wang et al., 1999; Andersson et al., 2000). Moreover, in patients with sepsis, increased serum HMGB1 levels correlated with mortality (Wang et al., 1999). Anti-HMGB1 antibodies have also been shown to be successful at attenuating the inflammatory response associated with APAP hepatotoxicity (Scaffidi et al., 2002). It has also been demonstrated that levels of serum HMGB1 in patients increased with APAP- and non-APAP-induced acute liver injury (Craig et al., 2011). These results suggest that HMGB1

is an important mediator in inflammation and APAP-induced liver injury and that its inhibition may be a key to improving clinical outcomes.

Ethyl pyruvate (EP) is a stable lipophilic pyruvate derivative of the endogenous metabolite pyruvic acid. The lipophilicity of EP thereby enables it to diffuse readily into cells, where it acts as a potent antioxidant and free-radical scavenger and has also been shown to be an effective anti-inflammatory agent in a variety of *in vitro* and *in vivo* model systems (Fink et al., 2007). EP has been shown to improve survival and ameliorate organ dysfunction in a wide variety of animal models of severe sepsis (Ulloa et al., 2002; Venkataraman et al., 2002), haemorrhagic shock (Yang et al., 2002b; Cai et al., 2009), hepatic ischaemia/reperfusion injury (Tsung et al., 2005), bacterial peritonitis (Ulloa et al., 2002; Su et al., 2007), and ethanol-induced acute liver injury (Yang et al., 2003). EP was shown to inhibit LPS-induced NF-κB activation in cultured RAW264.7 murine macrophage-like cells, and reduces HMGB1 release and TNF-α gene expression both *in vitro* and *in vivo* (Sims et al., 2001; Ulloa et al., 2002; Yang et al., 2002b).

To date there has not been a report of EP being used as an experimental pharmacological agent that could modulate APAP-induced hepatotoxicity and inflammation. Therefore, the overall aim of this chapter was to test the hypothesis that treatment with EP would be protective in our APAP model of hepatotoxicity and inflammation. We reasoned that EP might be protective in APAP-induced liver injury, depending on its inhibition of early (TNF-α, IL-6, IL-1β) and late (HMGB1) inflammatory cytokines. The aims of the studies in this chapter were to define if EP reduces hepatotoxicity in our model of APAP-induced hepatotoxicity as assessed by serum ALT activity and histological assessment, and if EP reduced inflammation in our APAP and LPS models, including modulation of HMGB1. The studies were designed to evaluate whether pretreatment or delayed treatment of EP could be beneficial in our mouse models of LPS and APAP hepatotoxicity associated inflammation. Pretreatment of EP was designed to test if its pharmacological effects were due to the inhibition of early cytokines, whereas delayed treatment of EP was tested to see if EP could modulate the late acting HMGB1 and see if it had an effect after the onset of toxicity and inflammation.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Infinity ALT liquid kits were purchased from ALPHA Laboratories (Eastleigh, U.K). HMGB-1neutralising antibody and control IgY antibody were from Shino-Test Corporation (Tokyo, Japan). Unless otherwise stated, all other chemicals and materials were purchased from Sigma-Aldrich (Poole, U.K).

4.2.2 Experimental animals

The protocols described were in accordance with criteria outlined in a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Animals were purchased from Charles River laboratories and had a 5 day acclimatisation period prior to experimentation. Animals were also maintained in a 12hr light/dark cycle with free access to food and water.

4.2.3 Animal dosing regime

Male C57BL/6 (18-23g) mice were either fasted overnight with free access to water or had free access to food and water prior to experimentation. Mice received an *i.p* injection of 0.9% saline or ethyl pyruvate (EP; sodium 130mM, potassium 4mM, calcium 2.7mM, chloride 139mM and EP 28mM, pH 7.0) 30min prior to administration of APAP (530mg/kg, *i.p.*) in 0.9% saline or with LPS (5mg/kg, *i.p.*) in 0.9% saline then were euthanized after 24hr. Control animals received 0.9% saline. Other groups of mice received an *i.p* injection of 0.9% saline or EP 4hr after administration of APAP (530mg/kg, *i.p.*) in 0.9% saline or LPS (5mg/kg, *i.p.*) in 0.9% saline and were then euthanized after 24hr. To determine the role played by circulating HMGB1 released after APAP treatment, male CD-1 mice (25-35g) were fasted overnight before intravenous administration of 200μg of neutralising chicken polyclonal antibody to HMGB1 or 200μg of a control IgY antibody. Antibody treatment was conducted 2hr after APAP administration (530mg/kg; *i.p*). Animals were then euthanized after 24hr as described by Antoine et al. (2010).

4.2.4 Assessment of hepatotoxicity in male C57BL/6 mice

Animals were sacrificed by CO_2 inhalation and cervical dislocation and blood was collected by cardiac puncture. Hepatotoxicity was assessed by serum ALT activity and histology as described in Chapter 2.

4.2.5 Determination of hepatic glutathione levels in C57BL/6 mouse whole liver during drug-induced hepatotoxicity

Total hepatic GSH levels were determined as described previously in Chapter 2. Levels of GSH were normalised to hepatic protein content using Bio-Rad protein assay reagent according to the manufacturer's instructions.

4.2.6 Histological examination of C57BL/6 mouse liver

Hepatotoxicity and leukocyte infiltration was assessed as part of the histological examination of HE-stained sections as described in Chapter 2. The assessment was undertaken by Prof A Kipar, Veterinary Pathology, School of Veterinary Science, University of Liverpool.

4.2.7 Measurement of cytokines in serum from animals dosed with LPS and APAP using Luminex Analysis

Serum was collected from control and treated animals. Concentrations of TNF-α, IL-6 and IL-1β were measured using the Bio-Plex Assay kit (Bio-Rad Laboratories, Inc) as described in Chapter 2. The lower range of the assay is <5.8 pg/ml for TNF-α, <0.74pg/ml for IL-6 and <10.36pg/ml for IL-1β. Levels below these values were assigned a value of zero.

4.2.8 Measurement of HMGB1 in serum from animals dosed with LPS and APAP

HMGB1 measurements were carried out using an ELISA according to the manufacturer's instructions. A chicken anti-HMGB1 antibody was used as a catcher antibody (1μg/well compared to known standards using 3,3',5,5'-tetramethyl-benzidine turnover by a peroxidise-linked anti-HMGB1 antibody. The limit of detection was 0.1ng/ml.

4.2.9 Statistical analysis

All results are expressed as mean ± standard deviation (S.D). Values to be compared were analysed for non-normality using a Shapiro-Wilk test. The unpaired t-test was used when non-normality was indicated. A Mann-Whitney U test was used for non-parametric data. Comparisons between multiple groups were performed with one-way ANOVA or, where appropriate, by two-way ANOVA, followed by a *post* hoc Bonferroni test. All calculations were performed using SPSS statistical software, results were considered significant when $p<0.05$.

4.3 RESULTS

4.3.1 Investigation into the effect of EP pretreatment on LPS-induced inflammation

A study by Ulloa et al., (2002) demonstrated that EP was an effective strategy to pharmacologically inhibit HMGB1 and could subsequently attenuate lethal systemic inflammation caused by endotoxaemia and sepsis. This study was repeated using the EP dose previously published (40mg/kg; Ulloa et al., 2002). An initial evaluation of EP as a therapeutic agent for inflammation and hepatotoxicity in our LPS model was conducted. C57BL/6 mice received a single dose of EP (40mg/kg; i.p.) followed 30min later by LPS (5mg/kg; i.p.) and then were euthanized after 24hr. Pretreatment with EP conferred a significant decrease of serum HMGB1 levels in LPS-dosed animals (701.32±162.43ng/ml) compared to mice treated with LPS alone (954.37±75.18ng/ml) (Figure 4.1). EP alone did not result in HMGB1 release (11.32±6.00ng/ml).

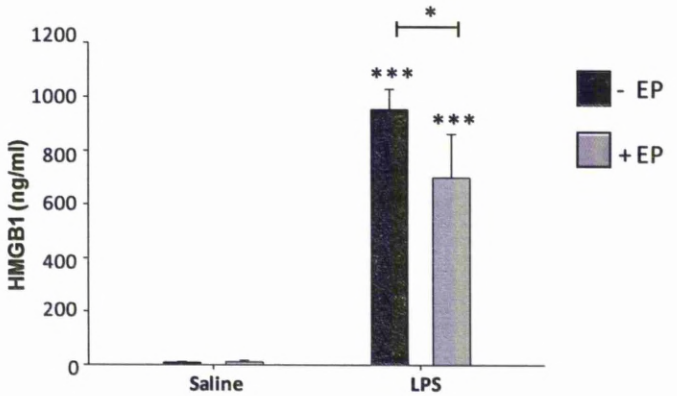

Figure 4.1: The effect of EP pretreatment on LPS-induced HMGB1 release
HMGB1 was measured in fasted male C57BL/6 mice pretreated with saline or EP (40mg/kg), 30min before administration of LPS (5mg/kg) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $*p < 0.05$, $***p < 0.001$.

Pretreatment with EP caused no hepatotoxicity as assessed by serum ALT activity (17.07±7.24U/l) (Figure 4.2A). LPS administered alone or with EP did not cause hepatotoxicity either (14.38±7.31U/l and 16.43±8.57U/l respectively). EP pretreatment did not alter hepatic GSH content compared to control (46.97±6.50nmol/mg), nor did LPS alone or with EP (45.12±5.95nmol/mg and 46.20±5.81nmol/mg respectively) (Figure 4.2B).

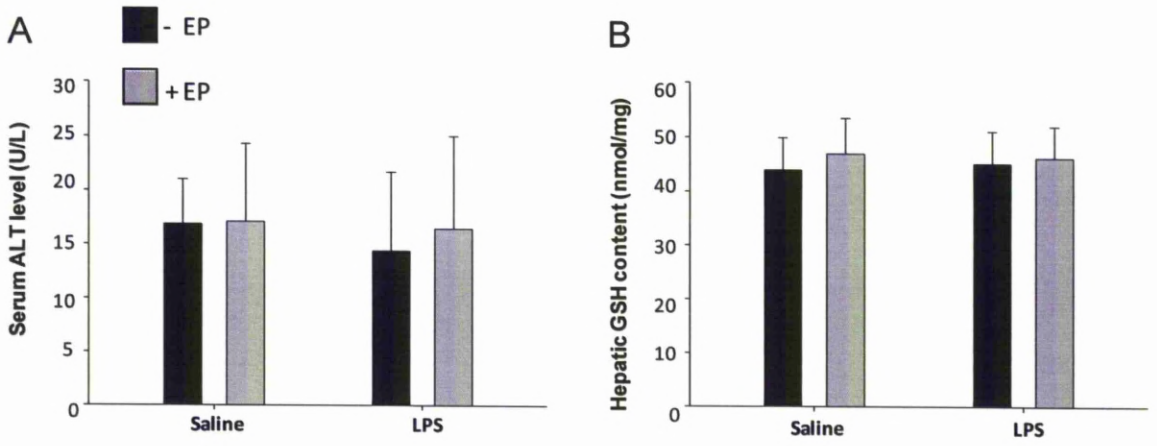

Figure 4.2: The effect of EP pretreatment on LPS-induced toxicity
Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice pretreated with saline or EP (40mg/kg), 30min before administration of LPS (5mg/kg) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 6 mice per group. Statistical significance was assigned relative to respective controls with or without EP.

EP pretreatment (40mg/kg) caused no cytokine release into the serum (Figure 4.3), whereas LPS (5mg/kg) caused a significant increase in the serum level of TNF-α (581.22±32.77pg/ml) (Figure 4.3A), the serum level of IL-6 (1825.57±342.23pg/ml) (Figure 4.3B) and the level of IL-1β (239.12±25.40pg/ml) at 24hr post LPS dose (Figure 4.3C). Pretreatment with EP significantly decreased serum levels of LPS-induced TNF-α (416.82±54.63pg/ml), IL-6 (1107.46±145.64pg/ml) and IL-1β (155.60±19.12pg/ml) after 24hr.

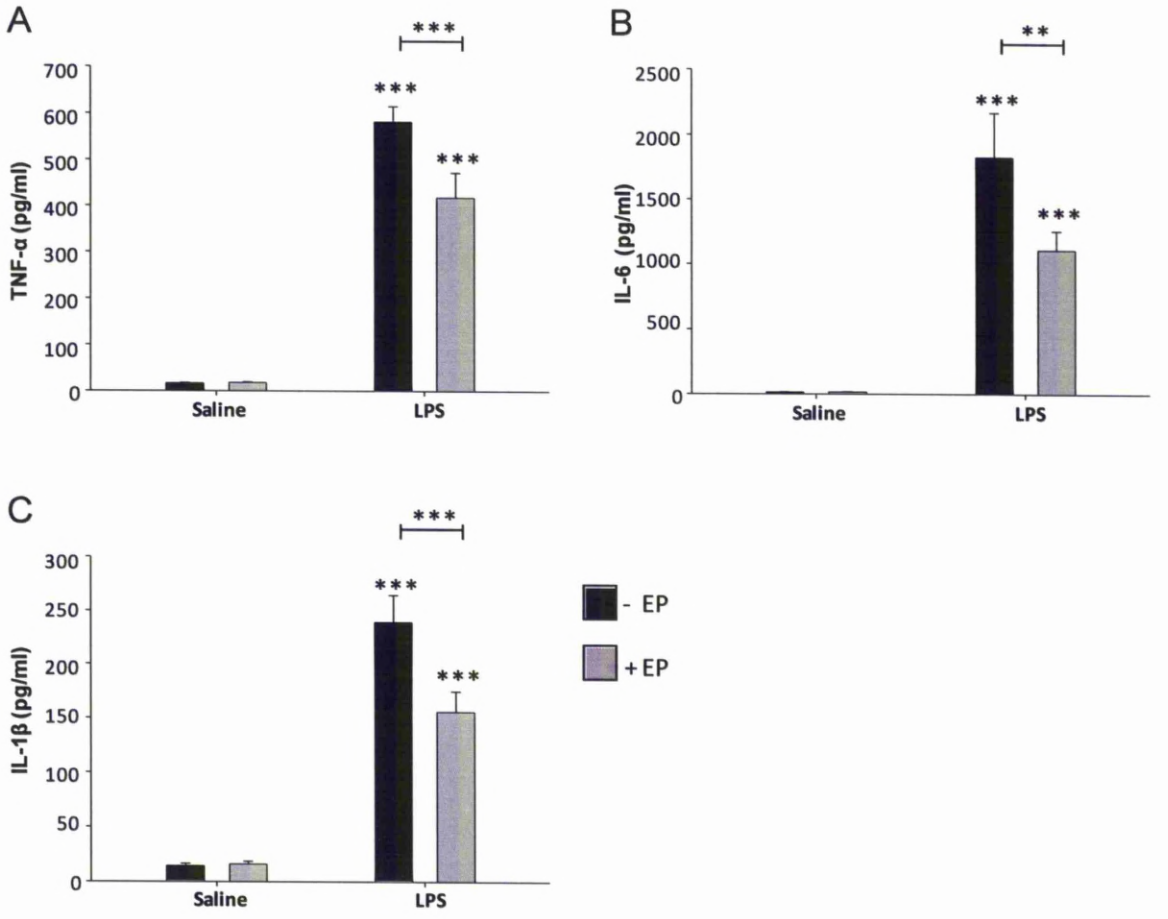

Figure 4.3: The effect of EP pretreatment on serum cytokine levels post LPS treatment
Fasted male C57BL/6 mice were pretreated with saline or EP (40mg/kg), 30min before administration of LPS (5mg/kg) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined at 24hr post treatment (- EP; black; + EP, grey). Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $^{**}p < 0.01$, $^{***}p < 0.001$.

The histological examination confirmed the biochemical findings. There were no findings indicating that EP had any effect on the liver. EP also appeared not to have an effect on the LPS-induced changes, i.e. neutrophil recruitment into the liver and individual hepatocyte death (Table 4.1).

Table 4.1: Histological findings after EP pretreatment of LPS treated mice. Male C57BL/6 mice were pretreated with saline or EP (40mg/kg) 30min before administration of LPS (5mg/kg). Animals were examined 24hr after LPS treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 6 animals per group.

Treatment	**Score***	**Histological findings**
Saline predose + Saline	0	Diffuse glycogen; no histological abnormality is recognised (NHAIR)
Saline predose + LPS	0	Diffuse glycogen; mild increase of neutrophils between hepatic cords; some small random neutrophil aggregates
EP predose + Saline	0	Diffuse glycogen; no histological abnormality is recognised
EP predose + LPS	0	No glycogen; mild increase of neutrophils between hepatic cords, some neutrophils in central veins and sinuses surrounding these; scattered small random neutrophil aggregates

* The scoring system for APAP-induced hepatotoxicity was applied (see Chapters 2 and 3).

4.3.2 Investigation into the effect of delayed administration of EP on LPS-induced inflammation

The therapeutic efficacy of EP when first administered after the onset of LPS-induced inflammation was assessed. Treatment with EP was initiated 4hr after LPS, a time when LPS-induced toxicity and inflammation would already be evident (as shown in Chapter 2; Figures 2.1 and 2.2). Delayed treatment of EP (40mg/kg) significantly reduced serum HMGB1 levels in LPS-dosed animals (790.77±136.60ng/ml) compared to mice treated with LPS alone (1055.92±176.48ng/ml) (Figure 4.4). EP alone did not result in HMGB1 release (11.65±5.10ng/ml).

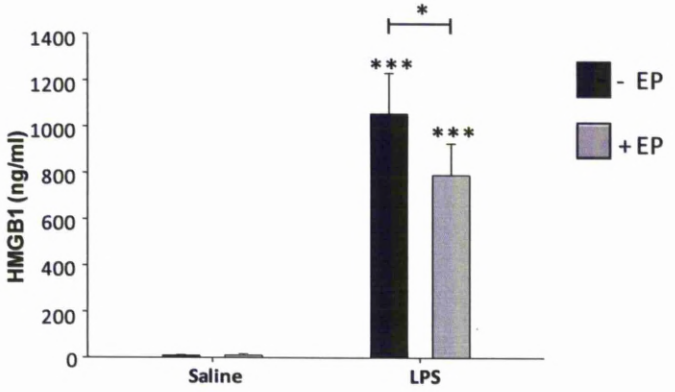

Figure 4.4: The effect of delayed administration of EP on LPS-induced HMGB1 release
HMGB1 was measured in fasted male C57BL/6 mice treated with saline or EP (40mg/kg), 4hr after administration of LPS (5mg/kg) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $*p < 0.05$, $***p < 0.001$.

Delayed treatment with EP (40mg/kg) caused no hepatotoxicity as assessed by serum ALT activity (9.14±2.20U/l) (Figure 4.5A). LPS administered alone or with EP did not cause hepatotoxicity either (26.76±12.16U/l and 27.15±7.94U/l respectively), although ALT was significantly higher than in controls. Delayed treatment with EP did not alter hepatic GSH content compared to control (44.52±3.75nmol/mg), nor did LPS alone or with EP (49.34±9.55nmol/mg and 48.64±3.02nmol/mg respectively) (Figure 4.5B).

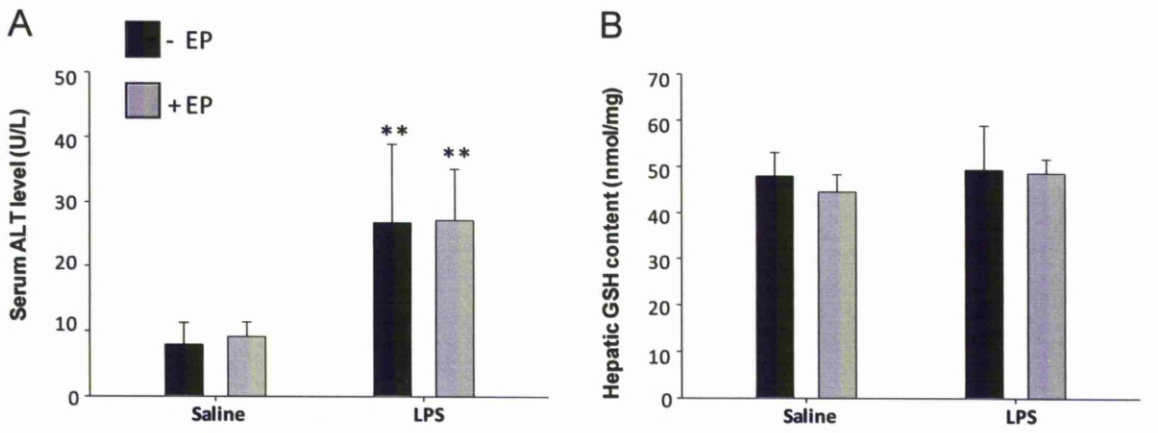

Figure 4.5: The effect of delayed administration of EP on LPS-induced toxicity
Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice treated with saline or EP (40mg/kg), 4hr after administration of LPS (5mg/kg) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 6 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $^{**}p < 0.01$.

Delayed treatment with EP (40mg/kg) caused no cytokine release into the serum (Figure 4.6). LPS (5mg/kg) caused a significant increase in the serum level of TNF-α (621.62±36.98pg/ml) (Figure 4.6A), IL-6 (1786.14±194.11pg/ml) (Figure 4.6B) and IL-1β (301.18±20.93pg/ml) (Figure 4.6C) at 24hr post LPS dose. Delayed treatment with EP did not significantly decrease serum levels of LPS-induced TNF-α (540.16±61.03pg/ml) or IL-6 (1504.02±286.19pg/ml) after 24hr whereas a significant reduction in IL-1β serum levels was observed (238.96±25.66pg/ml).

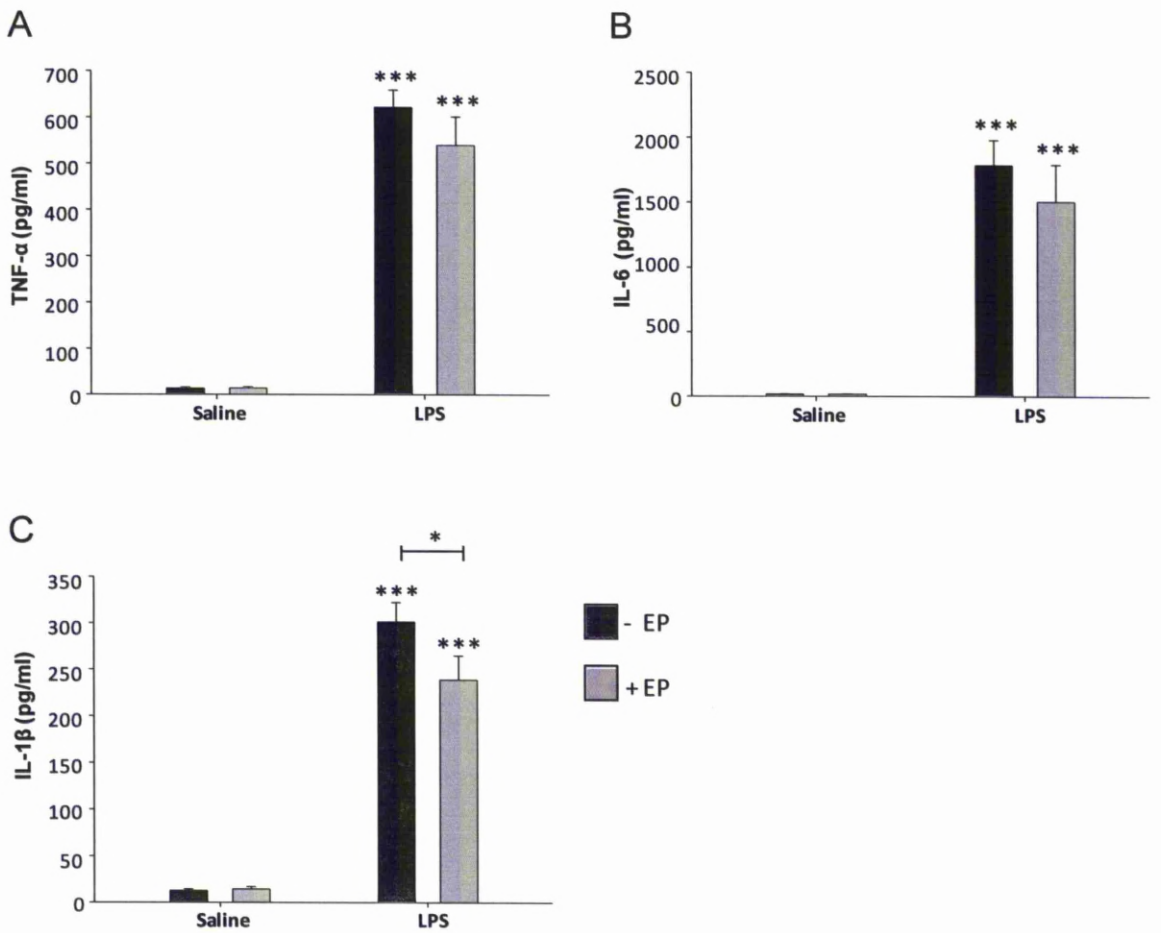

Figure 4.6: The effect of delayed administration of EP on serum cytokine levels post LPS treatment

Fasted male C57BL/6 mice were treated with saline or EP (40mg/kg), 4hr after administration of LPS (5mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined at 24hr post treatment. (- EP; black; + EP, grey). Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $*p < 0.05$, $***p < 0.001$.

The histological examination confirmed that delayed treatment with EP did not appear to have an effect on the LPS-induced changes, i.e. neutrophil recruitment into the liver and individual hepatocyte death (Table 4.2).

Table 4.2: Histological findings after delayed administration of EP to LPS treated mice. Male C57BL/6 mice were treated with saline or EP (40mg/kg) 4hr after administration of LPS (5mg/kg). Animals were examined 24hr after LPS treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 6 animals per group.

Treatment	**Score***	**Histological findings**
Saline + EP post dose	0	Diffuse glycogen; no histological abnormality is recognised
LPS + EP post dose	0	Diffuse glycogen (low amount); increased neutrophils between hepatic cords, some degree of central vein endothelial cell activation; several neutrophils within lumen of central veins and within sinuses surrounding central veins

* The scoring system for APAP-induced hepatotoxicity was applied (see Chapters 2 and 3).

4.3.3 Investigation into the effect of EP pretreatment on APAP-induced hepatotoxicity and inflammation

The efficacy of EP in reducing inflammation and toxicity in the APAP-model was tested next. Pretreatment with EP conferred a significant decrease of serum HMGB1 levels in APAP-dosed animals at 24hr post APAP (74.99±19.56ng/ml) compared to mice treated with APAP alone (125.27±30.17ng/ml) (Figure 4.7). EP alone did not result in HMGB1 release (11.32±6.00ng/ml).

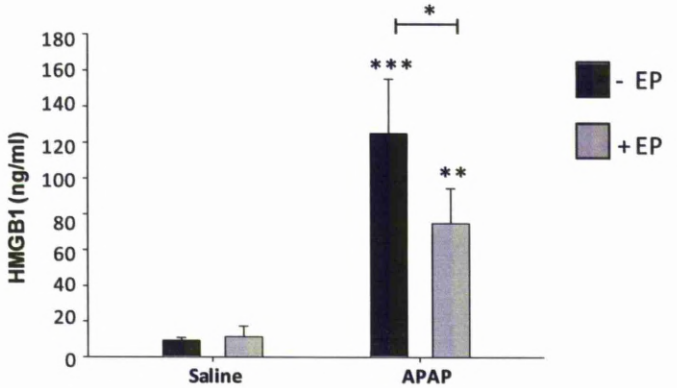

Figure 4.7: The effect of EP pretreatment on APAP-induced HMGB1 release
HMGB1 was measured in fasted male C57BL/6 mice pretreated with saline or EP (40mg/kg), 30min before administration of APAP (530mg/kg; 24hr) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Pretreatment with EP caused no hepatotoxicity as assessed by serum ALT activity (17.07±7.24U/l) (Figure 4.8A) nor did it reduce APAP hepatotoxicity compared to APAP-treated mice given a predose of saline (3307.72±501.05U/l and 3615.66±454.62U/l respectively). EP pretreatment did not alter hepatic GSH content compared to control (46.97±6.50nmol/mg). GSH content was lower in APAP-treated mice compared to control (35.51±4.03nmol/mg and 43.95±5.93nmol/mg respectively) although this reduction was not statistically significant. EP pretreatment did not cause any difference in GSH recovery after APAP overdose, with levels returning to control (38.12±5.58nmol/mg) (Figure 4.8B).

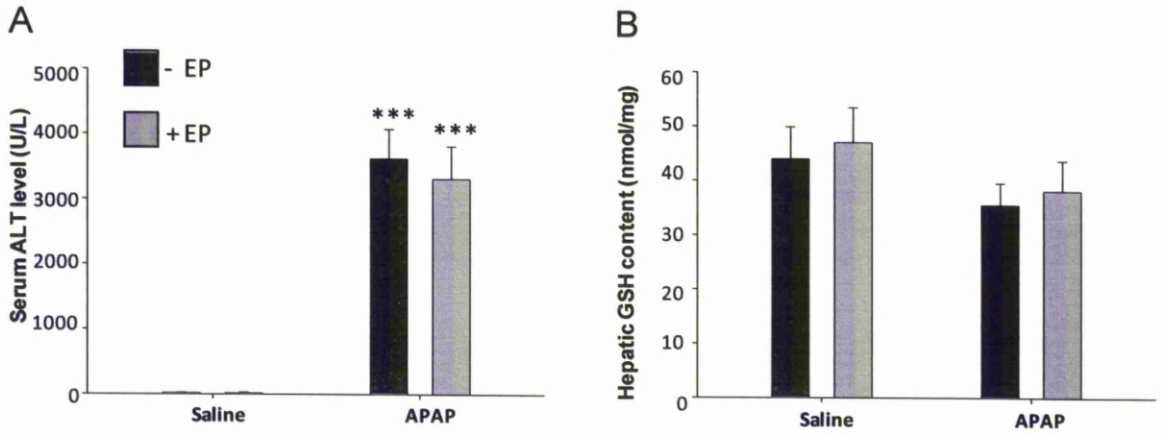

Figure 4.8: The effect of EP pretreatment on APAP-induced hepatotoxicity
Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice pretreated with saline or EP (40mg/kg), 30min before administration of APAP (530mg/kg; 24hr) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 6 mice per group. Statistical significance was assigned relative to respective controls with or without EP. ***$p < 0.001$.

EP pretreatment (40mg/kg) caused no cytokine release into the serum (Figure 4.9). APAP (530mg/kg) caused a significant increase in the serum level of TNF-α (292.41±32.68pg/ml) (Figure 4.9A), IL-6 (159.43±30.56pg/ml) (Figure 4.9B) and IL-1β (61.40±11.51pg/ml) (Figure 4.9C) at 24hr post APAP. Pretreatment with EP significantly decreased serum levels of APAP-induced TNF-α (215.96±17.46pg/ml) and IL-1β (28.15±7.26pg/ml) release after 24hr. Serum IL-6 levels were not reduced with EP pretreatment at 24hr post APAP (170.18±20.83pg/ml).

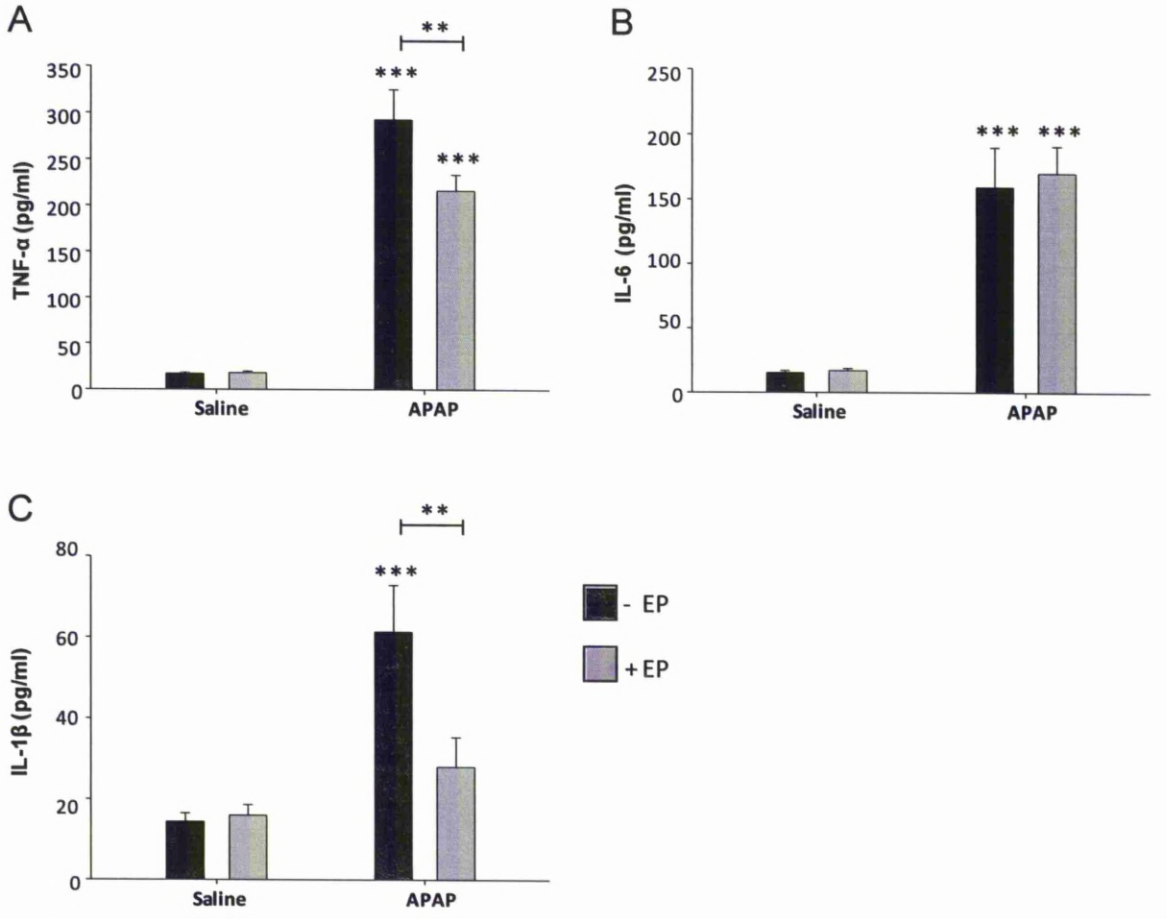

Figure 4.9: The effect of EP pretreatment on serum cytokine levels post APAP dose
Fasted male C57BL/6 mice were pretreated with saline or EP (40mg/kg), 30min before administration of APAP (530mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined at 24hr post treatment (- EP; black; + EP, grey). Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $^{**}p < 0.01$, $^{***}p < 0.001$.

The histological examination identified the typical APAP-induced changes in APAP-treated animals and confirmed that EP pretreatment does not have an effect on neutrophil recruitment into the liver after APAP-induced injury (Table 4.3).

Table 4.3: Histological findings after EP pretreatment on APAP treated mice. Male C57BL/6 mice were pretreated with saline or EP (40mg/kg), 30min before administration of APAP (530mg/kg). Animals were examined 24hr after APAP treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 6 animals per group.

Treatment	**Score* [average]**	**Histological findings**
Saline predose + Saline	0	Diffuse glycogen; no histological abnormality is recognised
Saline predose + APAP	2-5 [3.5]	No glycogen; centrilobular coagulative necrosis and apoptosis with hydropic degeneration of hepatocytes surrounding affected areas; some neutrophils in affected areas
EP predose + Saline	0	Diffuse glycogen; no histological abnormality is recognised
EP predose + APAP	2-5 [3.75]	No glycogen; almost diffuse hydropic degeneration (variable intensity); coagulative necrosis and apoptosis of remaining hepatocytes in affected areas; some neutrophils in affected areas

* The scoring system for APAP-induced hepatotoxicity was applied (see Chapters 2 and 3).

4.3.4 Investigation into the effect of delayed administration of EP on APAP-induced hepatotoxicity and inflammation

The efficacy of EP on APAP-induced hepatotoxicity and inflammation when administered after the onset of toxicity in the APAP model was tested. Delayed treatment with EP (40mg/kg) significantly reduced serum HMGB1 levels in APAP-dosed animals (97.28±20.40ng/ml) compared to mice treated with APAP alone (154.01±23.95ng/ml) (Figure 4.10). EP alone did not result in HMGB1 release (11.65±5.10ng/ml).

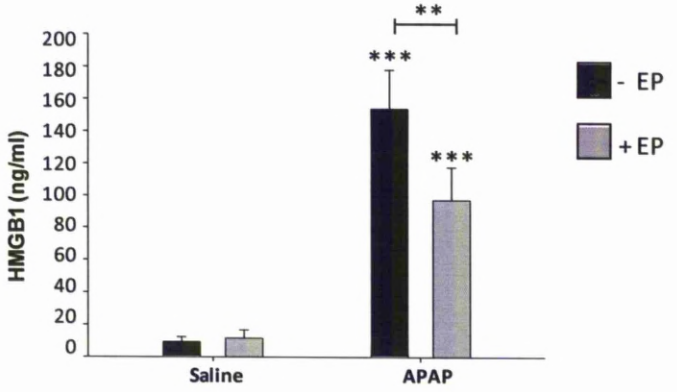

Figure 4.10: The effect of delayed administration of EP on APAP-induced HMGB1 release

HMGB1 was measured in fasted male C57BL/6 mice treated with saline or EP (40mg/kg), 4hr after administration of APAP (530mg/kg; 24hr) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to respective controls with or without EP. $^{**}p < 0.01$, $^{***}p < 0.001$.

Delayed treatment with EP (40mg/kg) caused no hepatotoxicity as assessed by serum ALT activity (9.14±2.20U/l) (Figure 4.11A). EP significantly reduced APAP hepatotoxicity compared to APAP treatment with a post dose of saline (2917.94±354.13U/l and 3441.89±380.05U/l respectively). Delayed treatment with EP did not alter hepatic GSH content compared to control (44.52±3.75nmol/mg). GSH content was significantly lower in APAP-treated mice compared to control (36.38±6.10nmol/mg and 47.96±5.06nmol/mg respectively). Delayed treatment with EP did not cause any difference in GSH recovery after APAP overdose, with decreased levels observed similar to APAP-alone (32.45±5.77nmol/mg) (Figure 4.11B). The histological examination showed that delayed EP treatment does not have an effect on APAP-induced hepatotoxic changes and neutrophil recruitment into the liver 24hr post treatment (Table 4.4).

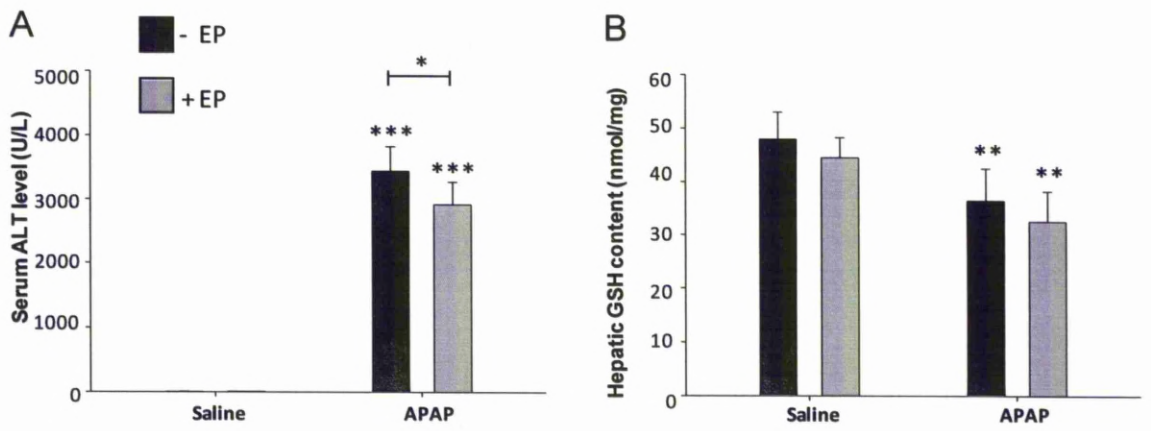

Figure 4.11: The effect of delayed administration of EP on APAP-induced hepatotoxicity

Serum ALT (A) and hepatic GSH content (B) were measured in fasted male C57BL/6 mice treated with saline or EP (40mg/kg), 4hr after administration of APAP (530mg/kg; 24hr) (- EP; black; + EP, grey). Mice were euthanized after 24hr. Data is given as mean ± SD of 6 mice per group. Statistical significance was assigned relative to respective controls with or without EP. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Delayed treatment with EP (40mg/kg) caused no cytokine release into the serum (Figure 4.12). APAP (530mg/kg) caused a significant increase in the serum level of TNF-α (286.05±17.52pg/ml) (Figure 4.12A), IL-6 (224.44±26.91pg/ml) (Figure 4.12B) and IL-1β (66.67±19.97pg/ml) (Figure 4.12C) 24hr post APAP dose. Delayed treatment with EP significantly reduced serum levels of APAP-induced TNF-α after 24hr (238.44±19.75pg/ml) whereas there was no significant reduction observed for IL-6 (190.70±17.02pg/ml) or IL-1β (46.67±10.25pg/ml).

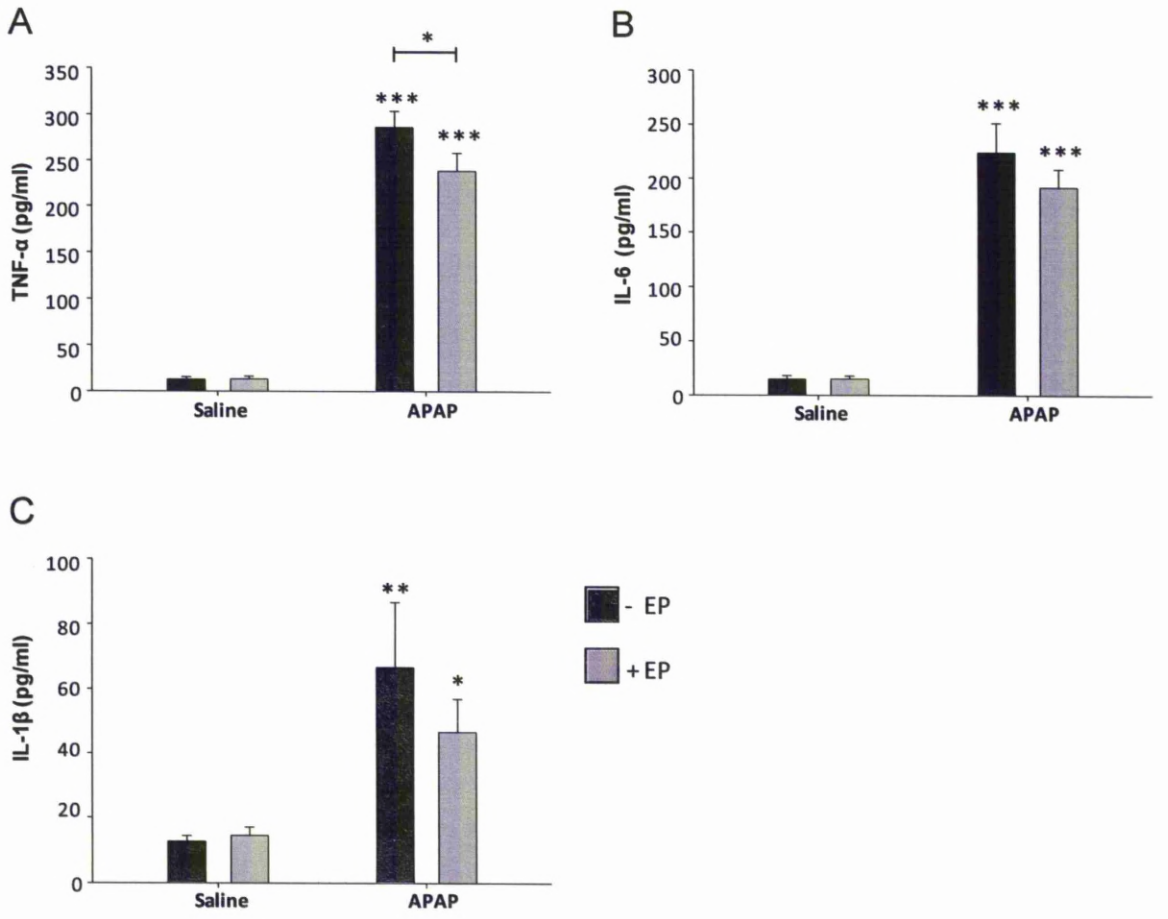

Figure 4.12: The effect of delayed administration of EP on serum cytokine levels post APAP dose

Fasted male C57BL/6 mice were treated with saline or EP (40mg/kg), 4hr after administration of APAP (530mg/kg; 24hr) and serum levels of TNF-α (A), IL-6 (B) and IL-1β (C) were determined at 24hr post treatment (- EP; black; + EP, grey). Data is given as mean ± SD of 3 mice per group. Statistical significance was assigned relative to respective controls with or without EP. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 4.4: Histological findings after delayed administration of EP on LPS treated mice. Male C57BL/6 mice were treated with saline or EP (40mg/kg), 4hr after administration of APAP (530mg/kg). Animals were examined 24hr after APAP treatment. The histological evaluation was undertaken by Prof Anja Kipar (Veterinary Pathology, School of Veterinary Science, University of Liverpool). The listed findings represent a summary of the changes observed in each of 6 animals per group.

Treatment	**Score* [average]**	**Histological findings**
Saline + EP post dose	0	Diffuse glycogen; no histological abnormality is recognised
APAP + EP post dose	2-4 [3]	Centrilobular coagulative necrosis and apoptosis; hydropic degeneration of hepatocytes surrounding affected areas; some neutrophils in affected areas; no glycogen or rim of glycogen loss around affected areas

* The scoring system for APAP-induced hepatotoxicity was applied (see Chapters 2 and 3).

4.3.5 Investigation into the effect of HMGB1 neutralisation on APAP-induced hepatotoxicity and inflammation

To enable the evaluation of a pharmacological inhibitor of HMGB1 as a modulator of inflammation and toxicity, a standard model with complete inhibition of HMGB1 was required. The antibody-mediated neutralisation of the inflammatory potential of HMGB1 has previously been reported (Scaffidi et al., 2002). This study was adapted and repeated in male CD-1 mice. Treatment of animals with a neutralising antibody to HMGB1 resulted in complete inhibition of serum HMGB1 levels after APAP challenge compared with that observed in IgY-treated animals (Figure 4.13A). There was significant reduction in the level of toxicity as assessed by serum ALT activity in the APAP + anti-HMGB1 antibody group compared with the APAP + control IgY group (Figure 4.13B). In both APAP-treated groups significant elevation in the serum level of TNF-α was observed (Figure 4.13C); however, the level of TNF-α was greater in the control group receiving APAP + IgY (232.3±54.2pg/ml) compared with the APAP + anti-HMGB1 group (121.0±16.7pg/ml). Mice treated with APAP + anti-HMGB1 also showed significant elevations in the serum level of IL-6 (246.2±36.8pg/ml) (Figure 4.13D).

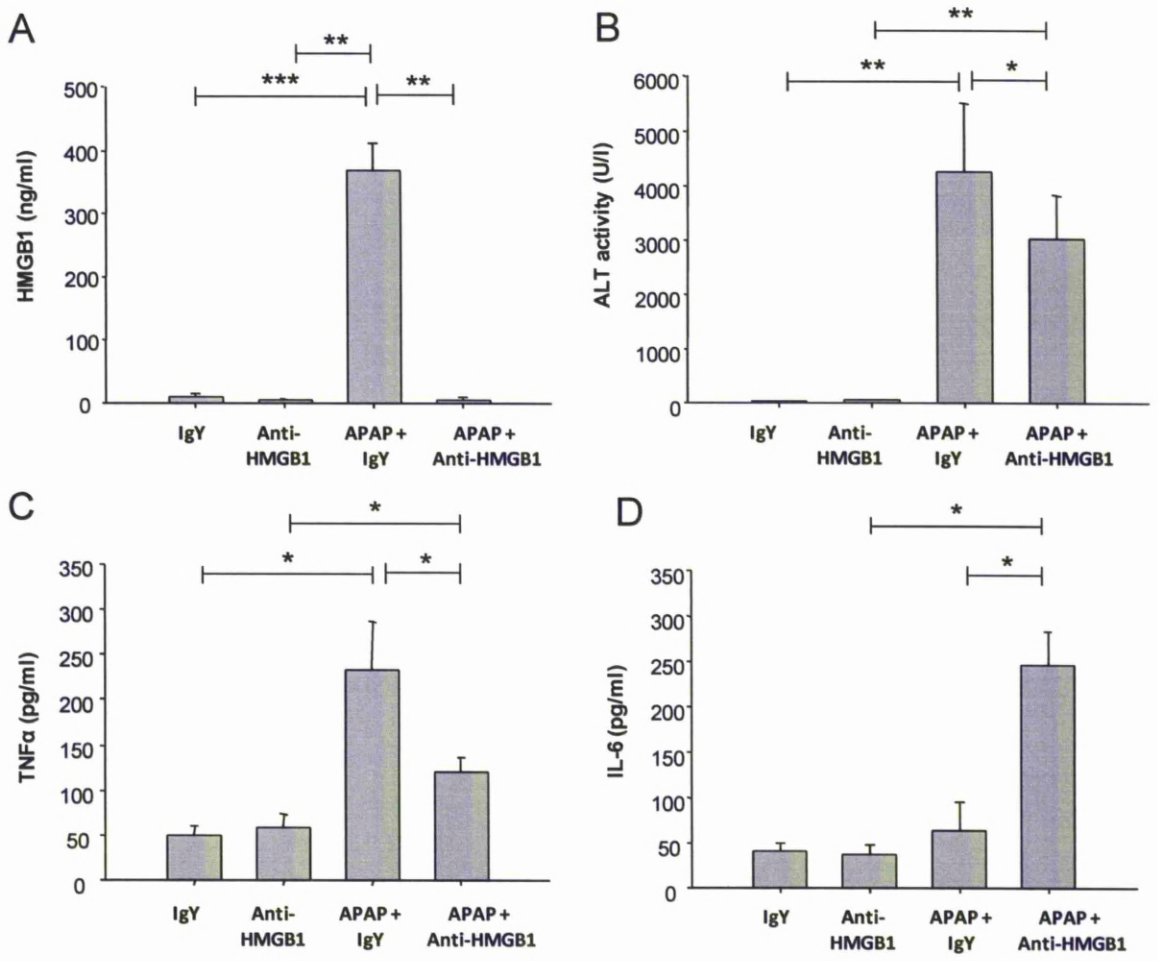

Figure 4.13: The effect of HMGB1 neutralisation on APAP-induced hepatotoxicity
Male CD-1 mice were treated with an HMGB1-neutralising antibody or a control IgY antibody 2hr after APAP (530mg/kg) treatment. Serum HMGB1 content (A), serum ALT activity (B), serum TNF-α (C) and serum IL-6 levels (D) were measured at 24hr post treatment. Data is given as mean ± SD of 6 mice per group. Statistical significance was assigned between groups as appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.4 DISCUSSION

EP has been shown to be an effective anti-inflammatory agent in a wide variety of in vivo and in vitro model systems of inflammation-mediated cellular or tissue injury, including severe sepsis, (Ulloa et al., 2002; Venkataraman et al., 2002), haemorrhagic shock (Yang et al., 2002; Cai et al., 2009), hepatic ischemia/reperfusion injury (Tsung et al., 2005), bacterial peritonitis (Ulloa et al., 2002; Su et al., 2007) and ethanol-induced acute liver injury (Yang et al., 2003). The molecular basis of EP action has previously been reported as interfering with signal transduction through the p38 MAPK and NF-κB signal transduction pathways and to target directly the p65 subunit of the transcription factor (Ulloa et al., 2002; Han et al., 2005). EP inhibition of the p38 MAPK and NF-κB signalling effectively prevents the release of early (TNF-α, IL-1β and IL-6) and late (HMGB1) inflammatory mediators. In a previous study by Ulloa et al. (2002), EP was shown to inhibit secretion of HMGB1 and improve the survival of mice in a model of endotoxaemia. The survival advantage was apparent when EP was administered 30min before LPS infusion, and when administered 24hr after the onset of disease.

The studies presented in this chapter were designed to test if EP can inhibit the release of both early (TNF-α, IL-1β and IL-6) and late (HMGB1) cytokines in our LPS and APAP models and subsequently, modulate the associated hepatotoxicity and inflammation. This was to be assessed alongside anti-HMGB1 antibody treatment, which offers a standard to which complete inhibition of HMGB1 is observed. Also, in both models a pre- and delayed treatment of EP was investigated to see if either treatment had a greater modulatory effect on hepatotoxicity and inflammation. In the LPS model of inflammation, the levels of both early and late cytokines in the serum were increased in mice 24hr after LPS administration compared to control mice. The results were in accordance with the findings in Chapter 2. Pretreatment with EP significantly inhibited the release of HMGB1 in LPS dosed animals although it is worth noting that this was not a complete inhibition compared to the level of inhibition observed with the anti-HMGB1 antibody. EP pretreatment also significantly reduced the serum levels of the cytokines TNF-α, IL-1β and IL-6 in LPS-treated mice. EP pretreatment had no effect on hepatotoxicity as assessed by serum ALT activity or hepatic GSH content. LPS alone did not cause hepatotoxicity or alter the level of GSH. Overall, the

early dose of EP significantly reduced both the early and late acting cytokines released after LPS administration.

Following the early treatment of EP, the therapeutic efficacy of EP was next assessed when administered after the onset of endotoxaemia and inflammation. Treatment with EP was initiated 4hr after the onset of endotoxaemia, a time at which clinical signs of LPS-induced toxicity, including diarrhoea, piloerection and depressed activity were already evident. Similarly to the outcome of pre-treating with EP, delayed administration led to a significant decrease in serum HMGB1 but not complete inhibition. However, delayed treatment only led to reduction that was statistically significant for IL-1β, not TNF-α or IL-6 serum levels. One possible explanation for this could be that EP was administered after an early peak in TNF-α and IL-6 levels, which has been seen to occur 1-3hr after the onset of endotoxemia (Hesse et al., 1988; Taudorf et al., 2007). The histological examination confirmed the biochemical findings. There were no findings indicating that pre- or delayed treatment with EP had any effect on the LPS-induced changes, i.e. neutrophil recruitment into the liver and individual hepatocyte death.

The pre- and delayed EP treatment regime was repeated in the APAP model of hepatotoxicity and inflammation. As reported in the literature, inhibition of HMGB1 using an anti-HMGB1 antibody modulated the inflammatory response associated with APAP. With HMGB1 partially inhibited by EP in our LPS model, which also led to modulation of cytokine levels, it was hypothesised that EP could protect against APAP-induced hepatotoxicity. Pretreatment with EP resulted in a significant decrease in HMGB1 post APAP-dose. The inhibition, as with the LPS model, was not absolute when compared to using anti-HMGB1 antibodies, however, a similar trend was observed regarding TNF-α and IL-6 serum levels. Both TNF-α and IL-1β serum levels were significantly lower in EP pretreated groups with APAP compared to APAP alone. IL-6 however, showed a slight increase although this was not statistically significant (Figure 4.9). The reduction of both early and late inflammatory mediators by pretreatment of EP suggests that EP had a prolonged effect after administration. This is supported by a study that showed pretreating mice with EP provided durable protection against some of the deleterious effects of LPS, even when the duration between the last dose of EP and the endotoxic challenge was 6hr (Sappington et al., 2003). Pretreatment with EP gave no protection in terms of APAP toxicity, as there was no significant decrease in

serum ALT activity compared to APAP alone (Figure 4.8). Further investigation is required to deduce why an early dose of EP did not protect against APAP hepatotoxicity. As EP is widely accepted as a potent antioxidant and free-radical scavenger (Varma et al., 1998; Sims et al., 2001), and that antioxidant treatment has been shown to protect against APAP hepatotoxicity (Oz et al., 2004), it could be hypothesised that early treatment of EP could protect via its antioxidant and free radical scavenger activities. The lack of protection is furthermore of interest when considering that EP is a lipophilic compound and thereby can diffuse readily into cells, potentially acting on both intracellular and extracellular radicals.

Delayed treatment with EP significantly reduced HMGB1 in APAP-treated mice compared to APAP alone. There was a trend showing a decrease in APAP-induced cytokine release but only TNF-α was statistically significant. Delayed treatment with EP, however, conveyed significant protection to APAP toxicity. As treatment with EP began after the initial toxicity, the results suggest that the significant protection conveyed by EP may be due to it targeting the late acting mediator of inflammation as observed by the significant reduction in HMGB1, rather than the earlier acting cytokines. Thus, overall these studies indicate that EP can significantly inhibit the release of both early (TNF-α, IL-1β and IL-6) and late (HMGB1) cytokines that mediate inflammation and toxicity. Furthermore, delayed treatment with EP can protect against APAP-induced hepatotoxicity. The histological examination showed that both pre- and delayed treatment of EP did not have an effect on APAP-induced hepatotoxic changes and neutrophil recruitment into the liver as there was no obvious difference in the degree of inflammatory cell recruitment into the liver.

HMGB1 is regarded as a late mediator of inflammation, because its release during endotoxaemia is delayed in comparison with the rapid increase of early pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Wang et al., 1999; Ulloa et al., 2002; Ulloa et al., 2003). The antibody-mediated neutralisation of the inflammatory potential of HMGB1 in a murine model of endotoxin lethality and protection has previously been reported (Wang et al., 1999). In this study HMGB1 increased in the circulation starting at 8hr, further increased until 16hr, and thereafter remained at a high level until 36hr during endotoxaemia. The administration of anti-HMGB1 antibodies reduced lethality, increasing the survival rate to 30% and conferring significant protection to a lethal dose of LPS. Likewise, we found that antibody neutralisation of HMGB1 in mice modulated the inflammatory response associated

with APAP hepatotoxicity. These findings are in accordance with data reported in a model of APAP hepatotoxicity by Scaffidi et al. (2002). Anti-HMGB1 administered 2hr after APAP completely abrogated the release of HMGB1 in mice, which was in parallel to a reduction in the level of hepatotoxicity as assessed by serum ALT activity and serum levels of TNF-α (Figure 4.13). However, the observed increase in IL-6 after anti-HMGB1 treatment with APAP requires further investigation. One possibility is that HMGB1 sequesters the hepatic IL-6 by direct protein-protein binding. The binding of HMGB1 and the alteration of cytokine function has previously been described (Sha et al., 2008). The delayed kinetics of actively released HMGB1 could provide a wider therapeutic window during which anti-HMGB1 antibodies or pharmacological inhibitors could be administered downstream of the initial cell damage and inflammatory response.

Investigating the level of protection afforded by completely inhibiting HMGB1 in APAP hepatotoxicity by using anti-HMGB1 antibodies allowed us to compare the effects of partially inhibiting HMGB1 by a pharmacological agent such as EP. The complete inhibition of HMGB1 by neutralising antibodies gave greater protection against APAP hepatotoxicity compared to partial inhibition of HMGB1 provided by EP. Moreover, a significant reduction in serum ALT was observed when EP treatment was delayed to a point when toxicity had already occurred.

Together, these results suggest that EP may have therapeutic potential for APAP-induced hepatotoxicity along with other potential conditions mediated by the release of the proinflammatory cytokines TNF-α, IL-1β and IL-6 and HMGB1. Although not absolute, EP is an effective inhibitor of HMGB1 release in our *in vivo* models of LPS and APAP hepatotoxicity and inflammation. EP warrants further evaluation as it may have a therapeutic potential in the clinic for inflammation associated with APAP-induced hepatotoxicity. EP has been tested in human volunteers and shown to be safe at clinically relevant doses (Fink, 2008). The consequence of HMGB1 signalling and its interaction with its receptors downstream of APAP-induced hepatotoxicity is another potential area of investigation to elucidate its role in the exacerbation of inflammation and toxicity.

CHAPTER FIVE

INVESTIGATION INTO THE EXPRESSION OF RECEPTOR FOR ADVANCED GLYCATION END PRODUCTS (RAGE) DURING ACETAMINOPHEN-INDUCED HEPATOTOXICITY

CONTENTS

5.1 INTRODUCTION

The immune system has evolved to respond not only to pathogens, but also signals released from dying cells. Severe injury to the liver, such as that induced by toxic doses of APAP, triggers a cascade of events leading to hepatic cell death. This initial cell stress or necrosis leads to the release of endogenous molecules such as S100 proteins, DNA and HMGB1 into the extracellular milieu. These molecules then act as Damage-Associated Molecular Pattern molecules (DAMPs), inducing activation of intracellular signalling pathways via interaction with at least three pattern recognition receptors; TLR-2, TLR-4 and the receptor for advanced glycation end products (RAGE) (Park et al., 2004).

The RAGE protein is a cell-surface molecule belonging to the immunoglobulin superfamily (Neeper et al., 1992). Its biological activity appears to be mainly dependent on the presence of its various ligands, including Advanced Glycation End products (AGEs) (Neeper et al., 1992; Schmidt et al., 1992), S100 calcium binding protein/calgranulins (Hofmann et al., 1999), amyloid-β-peptides (Du Yan et al., 1997) and HMGB1 (Hori et al., 1995), all known to be elevated in inflammatory diseases. RAGE is composed of three immunoglobulin domains located in the extracellular space, a single membrane spanning domain and a short cytoplasmic tail. RAGE exists as a full-length isoform (flRAGE) that is bound to the membrane and has several isoforms including soluble forms deriving from alternate splicing (known as endogenous secretory RAGE or esRAGE) or from proteolytic cleavage of the membrane-bound forms (soluble RAGE or sRAGE). The full-length form of RAGE contains the intracellular cytoplasmic domain, which is necessary for RAGE signalling. sRAGE lacks both the membrane and intracellular domains and appears to function as a decoy receptor that neutralises circulating ligands, subsequently preventing RAGE overactivation (Bucciarelli et al., 2002; Wendt et al., 2006). RAGE is now recognised as a key player in modulating the innate immune response. Ligand engagement of RAGE leads to prolonged inflammation by induction of NF-κB and MAPK kinases, resulting in RAGE-dependent expression of proinflammatory mediators such as cytokines and chemokines (Figure 5.1). RAGE-mediated signalling leads to the perpetuation of the initial signal both through *de novo* synthesis of p65 subunits of NF-κB and a positive feedback effect on RAGE expression (Schmidt et al., 2000; Bierhaus et al., 2001).

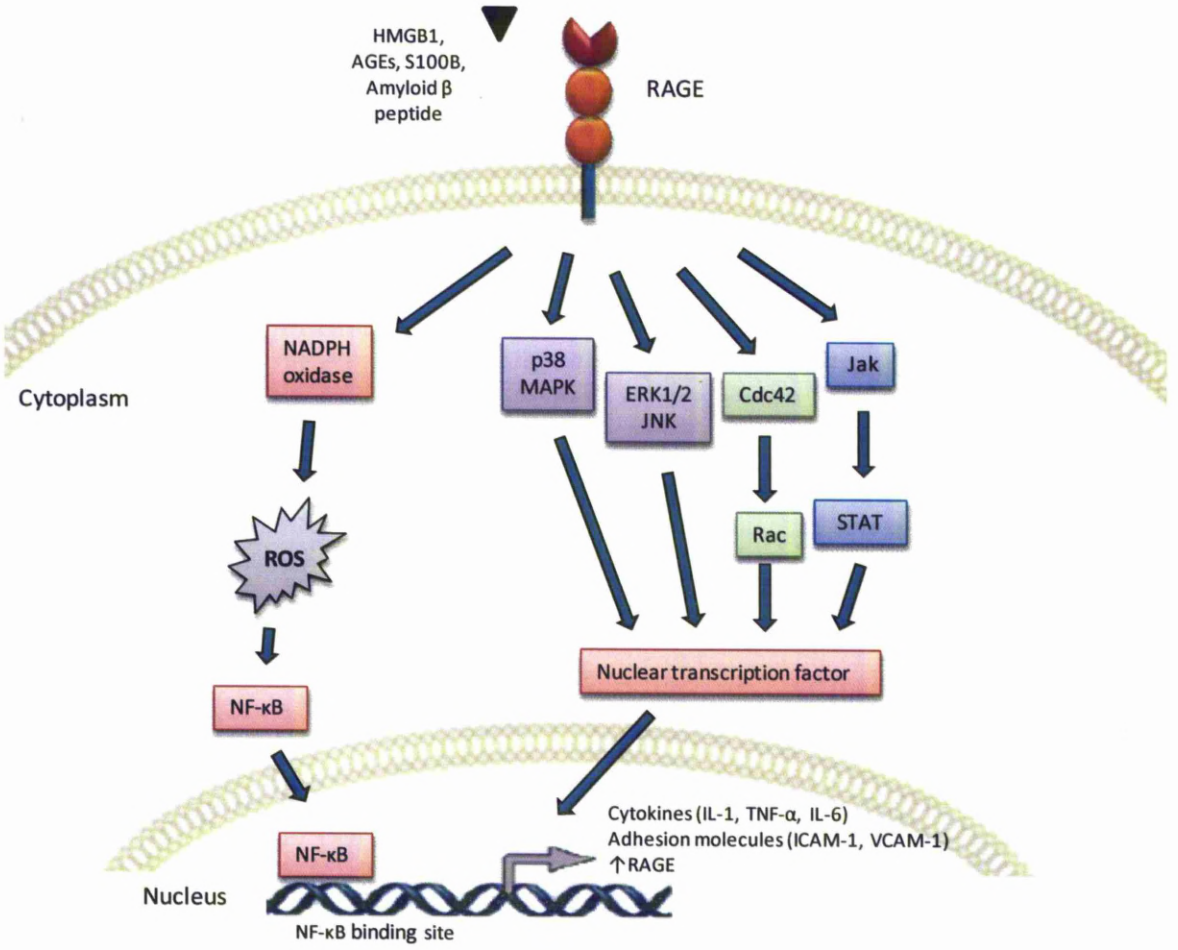

Figure 5.1: RAGE signal transduction pathways.
RAGE-induced signalling pathways. The engagement of ligands to RAGE results in the activation of a cascade of signalling pathways, including mitogen activated protein kinases (MAPK), such as extracellular regulated kinase (ERK)-1/2, p38 and c-Jun N-terminal kinase (JNK), Jak/STAT, and members of the Rho GTPase signalling pathway (Cdc42 and Rac-1), which converge to the activation of nuclear transcription factors, including nuclear factor (NF)-kB, subsequently leading to gene activation and cellular effects. Activation of RAGE also leads to increased oxidative stress (adapted from Xu et al., 2003).

RAGE is highly expressed and associated with many inflammation-based pathological diseases such as atherosclerosis (Park et al., 1998; Wendt et al., 2003), cancer (Taguchi et al., 2000), neurodegeneration (Yan et al., 1996; Yan et al., 1997) and diabetes (Wautier et al., 1996; Park et al., 1998b). The current opinion regarding the role of RAGE in the pathogenesis of inflammation associated conditions is that RAGE-mediated inflammation is deleterious and therefore RAGE inhibition may be potentially beneficial. In order to further

define a potential role of RAGE in immunity and inflammation RAGE signalling has been blocked or intercepted by using recombinant sRAGE and RAGE-deficient mice. Several studies investigating the role of RAGE in inflammatory diseases have used sRAGE to scavenge ligands in the extracellular space and prevent them from interacting with RAGE present at the cellular membrane (Hofmann et al., 1999; Taguchi et al., 2000; Hou et al., 2004). In animal models of hepatic ischaemia and massive liver resection, administration of sRAGE increased survival (Zeng et al., 2004; Cataldegirmen et al., 2005). The use of $RAGE^{-/-}$ mice has also been demonstrated to have a protective effect in a model of caecal ligation puncture (CLP), where $RAGE^{-/-}$ mice were better protected from the lethal effects of septic shock than wild-type controls (Liliensiek et al., 2004).

Experimental studies have given rise to the discussion that RAGE and its interaction with HMGB1 contribute to the pathophysiology of inflammatory conditions. It has been demonstrated in animal models that RAGE and HMGB1 play a key role in inflammation and oxidative stress-induced tissue injury, where the use of $RAGE^{-/-}$ mice, sRAGE, pharmacological inhibitors and antibodies or peptides targeted at RAGE or HMGB1 attenuate the lethal effects of endotoxin, ischaemia-reperfusion and APAP (Yang et al., 2004; Zeng et al., 2004; Kokkola et al., 2005; Ekong et al., 2006; Lutterloh et al., 2007a; 2007b; Zhang et al., 2008). The study by Ekong et al. (2006) looked at the effect of blocking RAGE using sRAGE on survival, generation of oxidative stress and expression of proinflammatory cytokines after APAP administration. They hypothesised that RAGE activation contributes to the generation of oxidative stress and inflammation within the livers of mice subjected to a lethal dose of APAP. They found that blockade of RAGE led to increased survival and a reduction in oxidative stress in APAP-treated animals.

The observations from Chapter 4 and in the literature that administration of anti-HMGB1 antibodies protected mice from sepsis lethality and APAP-induced hepatotoxicity (Wang et al., 1999; Scaffidi et al., 2002), and that blockade of RAGE could protect against APAP-induced hepatotoxicity (Ekong et al., 2006), suggests that the engagement of RAGE by HMGB1 may play an important role in mediating the pathogenic effects of HMGB1 during APAP-induced liver injury. Chapter 4 focused on the effect of inhibiting HMGB1 on APAP hepatotoxicity and inflammation, whereas the focus in this chapter is on the downstream signalling by HMGB1 via RAGE.

Despite reports that RAGE is upregulated in the presence of its ligands, to the best of our knowledge, no data is currently available for comparison between RAGE expression in APAP-treated livers and normal tissue. The aim of this chapter was to evaluate the expression of RAGE in the tissues of APAP-treated mice and its relationship to the pathophysiology of APAP hepatotoxicity at the mRNA, protein and tissue level. Our hypothesis is that RAGE is upregulated in response to danger signals such as HMGB1 that are released by necrotic cells during APAP-induced hepatotoxicity.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Infinity ALT liquid kits were purchased from ALPHA Laboratories (Eastleigh, U.K). Unless otherwise stated, all other chemicals and materials were purchased from Sigma-Aldrich (Poole, U.K). RAGE antibodies (RAGE H-300 and RAGE C-20) were purchased from Santa-Cruz Biotechnology.

5.2.2 Experimental animals

The protocols described were in accordance with criteria outlined in a licence granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Animals were purchased from Charles River laboratories and had a 5 day acclimatisation period prior to experimentation. Animals were also maintained in a 12hr light/dark cycle with free access to food and water.

5.2.3 Animal dosing regime

Male C57BL/6 mice (18-23g) were either fasted overnight with free access to water or had free access to food and water prior to experimentation. Mice (18-23g) were administered a single *i.p.* injection of APAP (530mg/kg) in 0.9% saline for 0-24hr. Control animals received 0.9% saline. Treatment groups consisted of 4 individual animals.

5.2.4 Isolation of total RNA from murine liver tissue

For each sample approximately 50-100mg of liver tissue was placed in an RNase free microtube (1.5ml). 200µl of Trizol Reagent was added to each microtube and using an eppendorf micropestle, the tissue was homogenised thoroughly. A further 800µl was added to each tube and the lysate was passed through a pipette tip to ensure homogenisation. The samples were then incubated at room temperature for 5min to permit dissociation of

nucleoprotein complexes. 200µl of chloroform was then added to each tube, after which each tube was vigorously shaken by hand for 15sec. The tubes were left to incubate for 2-3min at room temperature and then centrifuged for 15min (12,000 x g, 4°C) which separates the solution into an RNA-containing aqueous phase and an organic phase containing DNA and protein. 500µl of isopropyl alcohol was then added to the aqueous phase in a new tube and left to incubate at room temperature for 10min. After which, samples were centrifuged for 10min (12,000 x g, 4°C). The supernatant was discarded and 1ml of 74% EtOH was added to each tube, vortexed for 15sec and centrifuged for 5min (7,500 x g, 4°C). The supernatant was again discarded and the pellet was allowed to air dry for 5-10min. Finally, 44µl RNase-free H_2O was added to each tube to dissolve the pellet. The resultant RNA was stored at -80°C until DNase treatment could be performed.

5.2.5 DNase treatment of isolated RNA

DNA-free kit (Ambion) was used to eliminate DNA contamination in RNA extracts. To each tube containing 44µl of RNA, 5µl of 10x DNase buffer and 1µl of DNase I were added. The tubes were spun briefly (3,000rpm, 10sec) to reconstitute any bubbles and then placed on a heating block for 30min at 37°C. 5µl of DNase inactivation reagent was added to each tube and agitated gently to mix. The tubes were incubated for 2min at room temperature and then centrifuged for 1min (14,000rpm at room temperature). The resultant supernatant was transferred into new RNase-free tubes (0.6ml) and stored at -80°C until Reverse Transcription (RT) could be performed. An aliquot of supernatent was used to quantify RNA on an ND 1000 spectrophotometer (Nanodrop).

5.2.6 Reverse transcription of isolated RNA

The ImProm-II reverse transcriptase kit (Promega) was used to produce cDNA from total RNA. 2.5µg of DNA-free RNA was incubated with Oligo (dT16) and nuclease-free H_2O at 70°C for 5min. The samples were put on ice and the remaining RT components were added: 5x RT buffer, 25mM $MgCl_2$, dNTPs, reverse transcriptase and nuclease-free H_2O. The samples were then incubated in a thermocycler for 5min at 20°C, followed by 1h at 42°C,

then 15min at 70°C and held at 4°C. 200µl of nuclease-free H_2O was added to each tube and the cDNA was stored at -20°C until real time PCR could be performed.

5.2.7 Real time PCR of RAGE genes with SYBR Green

The mRNA sequence for RAGE was obtained at NCBI Nucleotide. Primers were designed using the Primer3 website and were blasted for specificity using the NCBI nucleotide blast database. Each primer set was designed to be between 18-22 bases, with a G/C content of 60%, a Tm of around 60°C and with the desired amplicon being between 100 and 180 bases. The sequences of the primer used for each gene were:

Endogenous secretory RAGE forward:	5'-GATGAGGGCACCTATAGCTGC-3'
Endogenous secretory RAGE reverse:	5'-TCCAGTCCCTCACCTTCAGC-3'
Total RAGE forward:	5'-GCTCTATGGGGAGCTGTAGC-3'
Total RAGE reverse:	5'-CAGTTTCCATTCTAGCTGCTGG-3'
Full length RAGE forward:	5'-AGGATGAGGAGGAACGTGC-3'
Full length RAGE reverse:	5'-GCTCTAGGGCCATCACACAG-3'
GAPDH forward:	5'-TGTCCGTCGTGGATCTGAC-3'
GAPDH reverse:	5'-CCTGCTTCACCACCTTCTTG-3'

The primers for each gene were custom synthesised by Eurofins MWG Operon. Real-Time PCR was performed using the SYBR Green JumpStart Taq ReadyMix kit (Sigma). Real-Time PCR reactions were set up in a thermo-fast 96-well detection plate to a final volume of 25µl. 10µg of cDNA was added to individual wells with 2 x SYBR Green, ROX, nuclease-free H_2O and 0.2µM of both forward and reverse primers for a specific gene. The thermocycler conditions of 95°C for 2min (1 cycle), 95°C for 15sec, 60°C for 1min (40 cycles) and held at 4°C. RAGE and GAPDH transcripts were quantified relative to GAPDH using SYBR green reagents (Sigma) and the ABI prism 7000 Sequence Detection System (applied biosystems). Dissociation curves were set up to confirm that primer products were

specific and that the SYBR Green fluorescence was a direct measure of accumulation of the product of interest.

5.2.8 Crude membrane isolation of murine whole liver tissue

Liver tissue (100mg) was washed twice in 1ml PBS then centrifuged at 2000rpm for 10min. The supernatant was discarded and the pellet was resuspended in hypotonic lysis buffer (10mM Tris pH 7.5, 10mM NaCl, 1mM $MgCl_2$, 1mM DTT and 1:500 protease inhibitor cocktail). The samples were frozen at -80°C then thawed out and left on ice (30-40min). The samples were then douce homogenised (50 strokes) and 2-fold hypotonic lysis buffer added. The samples were then spun down (500 x g, 10min, 4°C). The low speed supernatant was transferred into a centrifuge tube and disrupted with a 25 gauge needle and syringe (x 20). The membrane was collected by centrifugation (53,000rpm, 60min) and resuspended in 10mM Tris pH 7.5 and protease inhibitor cocktail.

5.2.9 RAGE immunoblotting in murine whole liver membrane

Membrane protein (5μg) was denatured via the addition of loading buffer containing 70% (v/v) NuPAGE sample loading buffer, 30% (v/v) NuPAGE reducing agent and was incubated at 80°C for 5min. Samples were loaded onto 4-12% NuPAGE bis-tris gels and resolved with 3-(N-morpholino)propanesulphonic acid (MOPS) and transferred to nitrocellulose membranes. Membranes were blocked with 10% (w/v) non-fat dried milk for 15min and then incubated overnight with a RAGE antibody (RAGE H-300, 1:2000 in 0.1% Tween-TBS with 2% (w/v) non-fat dried milk). Membranes were washed 3 times (5min/wash) with 0.1% Tween-TBS and incubated with an anti-rabbit secondary antibody conjugated to horse radish peroxidise for 1hr (1:10,000 in 0.1% Tween-TBS with 2% (w/v) non-fat dried milk). Immunoblots were visualised with western lighting chemiluminescence reagent and exposed to ECL Hyperfilm.

5.2.10 Immunohistology for RAGE

3-5µm thick paraffin-embedded liver sections were dewaxed in xylene (2 x 5min) and then submersed twice in 100% EtOH (2min) and again in 96% EtOH (2min). Endogenous peroxidise was blocked (360ml MeOH and 6ml H_2O_2, 30min) followed by two washes with water. For antigen retrieval, sections were then incubated with citrate buffer pH 6 for 30min in a water bath at 95°C then cooled on bench (15min), prior to a wash with dH_2O. Non-specific antibody binding was blocked by incubation with normal horse serum (10min), followed by overnight incubation with the primary antibody, goat polyclonal anti-mouse RAGE C-20 (1:200 in TBS; Santa-Cruz Biotechnology). The sections were washed in TBS for 5min and then a horse anti-goat secondary antibody (biotinylated anti-goat IgG, Vectastain) was added (0.9µl in 100µl TBS). After 30min incubation, sections were washed for 5min in TBS before incubation for 30min with ABC (Vectastain ABC-kit). The sections were washed for 5min in TBS, and then rinsed 4 times in dH_2O before incubation with diaminobenzidine hydrochloride (DAB; Fluka Analytical) for 10min to develop. After incubation, the sections were washed in dH_2O (3 x 5min) and then counterstained with Papancolaou's haematoxylin (Merck) for 1min. The sections were rinsed under running tap water for 5min then dehydrated and cleared by a series of steps (96% EtOH for 1min, 100% EtOH for 2min, 100% EtOH for 3min, xylene for 2min, xylene for 3min, xylene for 3min), then mounted with DPX (BHD). Sections of lung from a control mouse served as a positive control for RAGE expression. Negative controls were represented by consecutive sections that were incubated without the primary antibody.

5.2.11 RAGE RNA *in situ*-hybridisation

5.2.11.1 Polymerase chain reaction (PCR) for amplification of RAGE DNA

A 517-bp fragment for RAGE DNA was amplified with
RAGE forward (GAGGGAAGGAGGTCAAGTCC) Tm 61.4, GC 60% and
RAGE reverse (GGTGCACCATCCTTTATCCA) Tm 57.3, GC 50%
PCR was used to amplify DNA fragments for cloning. For cloning purposes the Expand High Fidelity^{PLUS} PCR System (Roche) was used. 50µl PCR mix contained 5µl of 10x buffer 2, 1µl of 10µM dNTPs, 2µl of both forward and reverse primers (10µM stock) and 2.5µl of template cDNA (50ng stock).

5.2.11.2 Ligation of RAGE DNA into vector

Ligation of the insert/PCR product into pCRII-TOPO vector (Invitrogen) was carried out with a 4:1 insert-vector ratio. In a total reaction volume of 3µl, 2µl of insert, 0.5µl of vector and 0.5µl of salt solution was used. The ligation was carried out at room temperature for 30min.

5.2.11.3 Cloning and transformation of RAGE DNA vector in bacteria

One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) were used for cloning and transformation purposes. Transformation of competent *E. coli* cells was carried out by thawing three tubes containing 50µl of cells on ice and adding the entire ligation mix (3µl) to one tube, 0.5µl of TOPO-vector to another and 0.5µl of pUC18 vector (positive control) to the third tube. The cells were gently shaken by hand before incubating on ice for 30min, heat shocked at 42°C for exactly 30sec and put on ice for 1min before 250µl of SOC medium was added to the tube. The cells were incubated at 37°C for 1hr at 250rpm in a shaking incubator, before 50µl and 150µl were plated on agar plates containing 100µg/ml ampicillin and incubated at 37°C overnight. Colonies represent bacteria with ampicillin resistance and sufficient ligation of PCR product. Discrete colonies were dabbed with filtered pipette tips which were then dropped into 20ml sterilin tubes containing 2ml LB broth and supplemented with 1µg/ml ampicillin. The tubes were incubated at 37°C at 250rpm overnight in a shaking incubator. Small yield plasmid DNA preparations from 1.5ml bacterial cultures (Mini-preparations) were done with the QIAprep spin Miniprep Kit (Qiagen) as per manufacturer's instructions, to obtain high quality DNA. Large yield plasmid DNA preparations from 150ml bacterial cultures (Maxi-preparations) were done with the Qiagen plasmid Maxi Kit to prepare stocks of plasmid DNA.

5.2.11.4 Restriction enzyme digestion of RAGE DNA

Plasmid DNA was linearised by restriction enzyme digestion. Two separate reactions were set up using two different restriction enzymes for sense and antisense riboprobes, *EcoRV* (cut site 5'-GAT^ATC-3') and *Sac*I (cut site 5'-GAGCT^C-3') respectively. For each reaction 5µg plasmid DNA was added to 5µl of the appropriate restriction enzyme, 5µl of 10x

restriction enzyme buffer and made up to a total volume of 50µl with ddH_2O. The reaction mix was incubated at 37°C for 2hr.

5.2.11.5 Phenol-chloroform extraction and ethanol precipitation of RAGE DNA

To remove salts and protein, template DNA was subjected to phenol-chloroform extractions followed by ethanol precipitations. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA templates. The tubes were then vortexed vigorously and centrifuged at 14,000rpm at room temperature for 1min. The upper aqueous phase was transferred into a new tube and the steps repeated once more. An equal volume of chloroform:isoamyl was added and the tubes were again vortexed vigorously and centrifuged at 14,000rpm at room temperature for 1min with a fresh tube used for the upper aqueous phase. The DNA was precipitated with 1/10 volume of 3M NaOAc and 3x volume of ice cold 100% EtOH. DNA was then precipitated by incubating at -20°C for 1hr or overnight followed by centrifugation for 10min (14,000rpm, room temperature). The supernatant was discarded and the pellet was washed with an equal volume of 70% EtOH. The tube was centrifuged for 10min (14,000rpm, room temperature), before the remaining EtOH was removed. The pellet was air-dried for 10min and then dissolved in 30µl of MQ H_2O.

5.2.11.6 RAGE RNA probe preparation

The labelling of RNA was done by *in vitro* transcription using digoxigenin-11-UTP as the label molecule. 1µg of linearised template DNA was added to an RNase-free tube and MQ H_2O was added to make a final volume of 13µl. DNA templates were mixed with 10x transcription buffer, RNase inhibitor, 10x NTP labelling mixture (10mM ATP, CTP, GTP, 6.5mM UTP, 3.5mM Digoxigenin-11-UTP (pH 7.5)) and SP6 (sense) or T7 (antisense) RNase polymerase. The mixture was centrifuged briefly at 5,000 x g and then incubated at 37°C for 2h. 2µl RNase-free RQ1 DNase was added with 1µl RNase inhibitor to digest the DNA template to leave purified RNA probes, and the mixture was further incubated at 37°C for 15min. The reaction was stopped by adding 0.2M EDTA. 1mg/ml of yeast tRNA was added to the DNase digested probes, and the RNA was precipitated by addition of 1/10 volume of 3M NaOAc pH 5.2 and 2.5x volume 100% EtOH. The mixture was incubated at -

80°C for 30min then spun at room temperature for 20min. The supernatant was discarded and the pellet was washed with 70% EtOH followed by centrifugation for 5min at room temperature. The supernatant was then carefully removed and the pellet was air-dried for 10min before being resuspended in 45µl of nuclease-free H_2O. Alkaline hydrolysis was then used to generate a shorter probe from 500bp to 350bp. 5µl of 0.4M $NaHCO_3$/0.6M Na_2CO_3 pH 10.2 was added to the probe and incubated for exactly 7min 48sec at 60°C. The mixture was neutralised by adding 5µl of 3M NaAc pH 4.6 and the previous ethanol precipitation and wash step was repeated. The pellet was air-dried for 10min before being resuspended in 50µl nuclease-free H_2O. To test the efficiency of DIG labelling of the newly synthesised probe as series of dilutions were prepared using yeast tRNA as the diluent (1:10-1:100,000). 5µl of each dilution was spotted onto a nylon membrane (Amersham) and allowed to dry before cross linking using a UV Stratalinker. The membrane was incubated on a shaker in washing buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5, 0.3% v/v Tween 20) for 2min and then blocked in blocking solution (1x blocking solution in maleic acid buffer) for 30min. The membrane was then incubated with an anti-digoxigenin antibody (Anti-DIG-AP, 1:5000 in blocking solution) for 30min. It was rinsed twice for 15min in washing buffer and then a further 3min in detection buffer (0.1M Tris HCl, 0.1M NaCl, pH 9.5), followed by incubation. The membrane was then incubated in 10ml of colour substrate BCIP/NBT tablets (1 per 10ml) in the dark without shaking until the colour precipitate formed and reached the required concentration. The reaction was stopped using 50ml TE buffer (10mM Tris-HCl, 1mM EDTA pH 8).

5.2.11.7 Pretreatment of murine liver tissue sections

In order to improve signal and reduce background, sections were subjected to several pretreatment steps before the hybridisation probes were applied. 3-5µm thick paraffin-embedded liver sections were prepared on polylysine coated slides. They were dewaxed in xylene (2 x 5min) and then submersed twice in 100% alcohol (5min) and again in 96% alcohol in DEPC ddH_2O (1 x 5min) followed by 70% alcohol in DEPC ddH_2O (1 x 5min). The sections were then submersed in an autoclaved glass tank containing ddH_2O with DEPC (5min) before being transferred into an autoclaved coplin jar containing ddH_2O with DEPC (1min). The sections were washed in 1x PBS for 5min and then left to stand for 20min at room temperature in 0.2M HCl. After, the sections were washed twice with 2x SCC plus

5mM EDTA at 50°C for 30min each and then incubated with proteinase K (1M Tris, 0.1M $CaCl_2$, ddH_2O with DEPC, proteinase K) at 37°C for 15min. The proteinase K reaction was stopped by washing slides in 0.2% glycine in PBS at room temperature for 5min. Slides were immersed in 4% PFA at room temperature for 4min and then washed twice in 1x PBS for 1min. Slides were washed in 1x PBS plus 5mM $MgCl_2$ at room temperature for 15min. The sections were immersed into 0.25% acetanhydride in 0.1M triethanolamide (pH 7.5) for 10min and then rinsed twice in 1x PBS at room temperature for 1min each before washing in 1x PBS at room temperature for 15min.

5.2.11.8 Hybridisation of RAGE riboprobe to murine liver tissue

Sections were treated with prehybridisation buffer-mix (prehybridisation buffer, RNA, ssDNA) for 1 hr at 52°C. The digoxigenin-labelled riboprobe was diluted 1:100 in hybridisation buffer-mix (hybridisation buffer, RNA, ssDNA, dextransulphate). 40µl of probe/hybridisation mixture was added on each slide under a plastic coverslip to prevent evaporation then placed in a hybridisation chamber and incubated overnight at 37°C to allow the probe to hybridise to targets. The coverslip was removed and the sections were washed with a series of SCC with decreasing concentrations to further remove unbound probes. For this purpose the sections were rinsed twice in 6x SCC plus 45% formamide at 42°C for 15min at a time then twice in 2x SCC at room temperature for 5min each and then washed twice in 0.2x SCC at 50°C for 15min each.

5.2.11.9 Staining of RAGE riboprobe signals

The slides were incubated in Buffer 1 (100mM Tris, 100mM NaCl, ddH_2O, pH 7.5) for 1min. To prevent non-specific binding, sections were blocked in blocking solution (NSS, 10% triton X-100, Buffer 1) at room temperature for 30min. Slides were then incubated in blocking solution containing AP-conjugate antibody (Anti-DIG-AP) at the concentration of 1:200 at room temperature for 2hr. After incubation with antibody, sections were washed twice with Buffer 1 at room temperature for 15min before washing in Buffer 3 (100mM Tris, 100mM NaCl, 50mM $MgCl_2.6H_2O$, ddH_2O, pH 9.5) for 2min. The sections were then incubated with staining solution (Buffer 3 containing NBT, X-phosphate, levamisole). The development of

the sections was followed using a microscope. The reaction was stopped by submerging the sections in Buffer 4 (10mM Tris, 1mM EDTA, ddH_2O, pH 8) and rinsing a few times in ddH_2O for 5min. The sections were then stained using Papanicolaou's haematoxylin counterstain for 10sec before bluing in tap water for 5min. The slides were dried by blotting on a clean paper towel before adding a drop of prewarmed (37°C) glycer-gel to the slides and covered using a coverslip. The slides were left to dry in the dark until analysis.

5.2.12 Statistical analysis

All results are expressed as mean ± standard deviation (S.D). Values to be compared were analysed for non-normality using a Shapiro-Wilk test. The unpaired t-test was used when non-normality was indicated. A Mann-Whitney U test was used for non-parametric data. Comparisons between multiple groups were performed with one-way ANOVA followed by a *post* hoc Bonferroni test. All calculations were performed using SPSS statistical software, results were considered significant when $p<0.05$.

5.3 RESULTS

5.3.1 Quantification of RAGE mRNA expression during APAP-induced hepatotoxicity

The expression of RAGE in the hepatic tissue of APAP-treated C57BL/6 mice was first evaluated at the mRNA level. Mice were either fasted or fed prior to administration of APAP (530mg/kg; 0-24hr). Purified mRNA from mouse lung encoding total RAGE as well as a full-length (flRAGE) and endogenous secretory isoform (esRAGE) was identified in RNA extracts from mouse livers and quantified using RT-PCR. RT-PCR analysis revealed a significant 3-fold increase of total RAGE mRNA post-APAP in fed mice at 3hr compared to the corresponding time-matched control (Figure 5.2A). In fasted mice, total RAGE mRNA increased after APAP-dose as early as 1hr (4-fold) and peaked at 5hr with an 8-fold increase over control levels (Figure 5.2B). In both fasted and fed mice, total RAGE mRNA levels had returned to normal by 24hr.

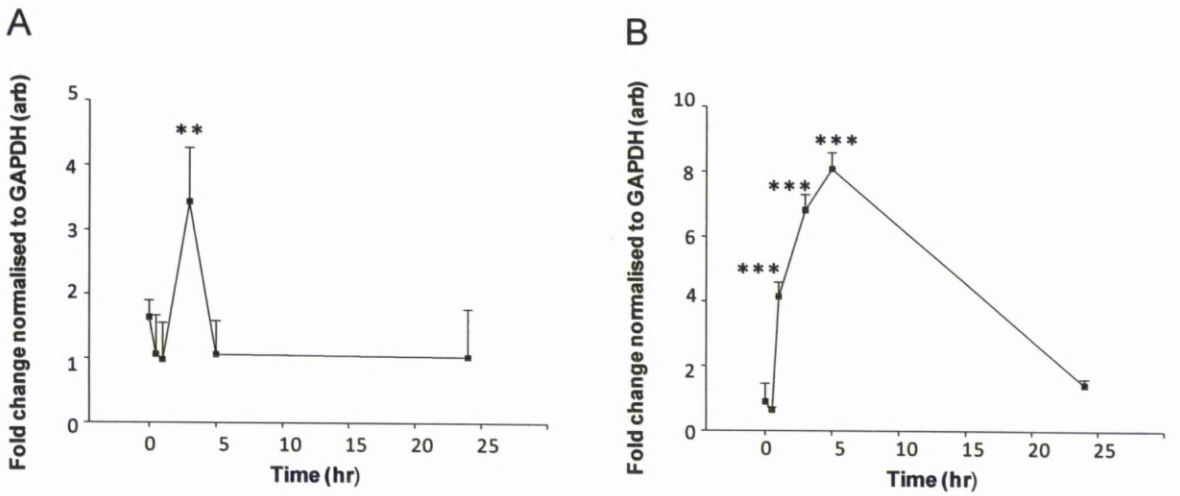

Figure 5.2: Total RAGE mRNA expression in response to APAP-induced hepatotoxicity in fed and fasted mice

The level of total RAGE mRNA expression was assessed by RT-PCR. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 0-24hr) or 0.9% saline. Results were normalised to the house-keeping gene, GAPDH and expressed relative to time-matched saline controls, using the comparative Ct method. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to time-matched controls. $^{**}p < 0.01$, $^{***}p < 0.001$.

The hepatic mRNA expression of the full-length RAGE isoform was determined next. In fed mice, flRAGE significantly increased 2.5-fold compared to saline control at 3hr and returned to control levels at both 5hr and 24hr time points (Figure 5.3A). In contrast to the findings in fed mice, flRAGE mRNA in fasted animals significantly increased at the earlier time point of 1hr (2.5-fold), continued to increase at 3hr (4.8-fold) and peaked at 5hr (5.2-fold) before returning to control levels at 24hr (Figure 5.3B).

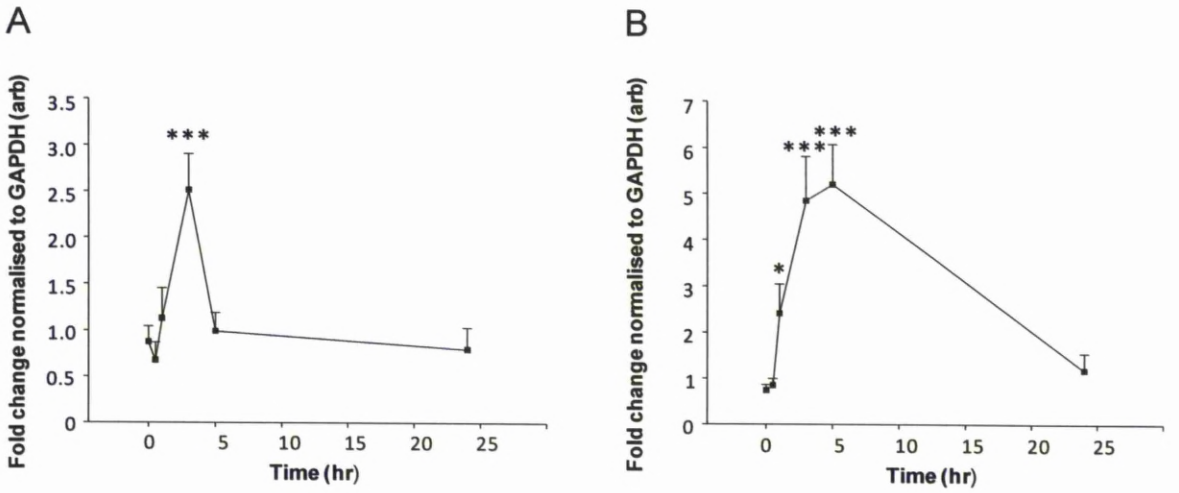

Figure 5.3: Full-length RAGE mRNA expression in response to APAP-induced hepatotoxicity in fed and fasted mice

The level of full-length RAGE mRNA expression was assessed by RT-PCR. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 0-24hr) or 0.9% saline. Results were normalised to the house-keeping gene, GAPDH and expressed relative to time-matched saline controls, using the comparative Ct method. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to time-matched controls. $*p < 0.05$, $***p < 0.001$.

The final RAGE gene that was evaluated was the endogenous secretory isoform. In fed mice, there was a significant 1.8-fold increase in esRAGE mRNA at 1hr compared to saline control (Figure 5.4A). At 3hr, esRAGE had further increased and peaked (2.3-fold) before returning to control levels at 5hr and 24hr. In fasted mice, esRAGE mRNA also increased at 1hr, however, the increase was 2.5-fold greater when compared to fed mice (4.7-fold) (Figure 5.4B). esRAGE mRNA further increased at 3hr (6.2-fold) and peaked at 5hr (11.2-fold), before returning to control levels at 24hr.

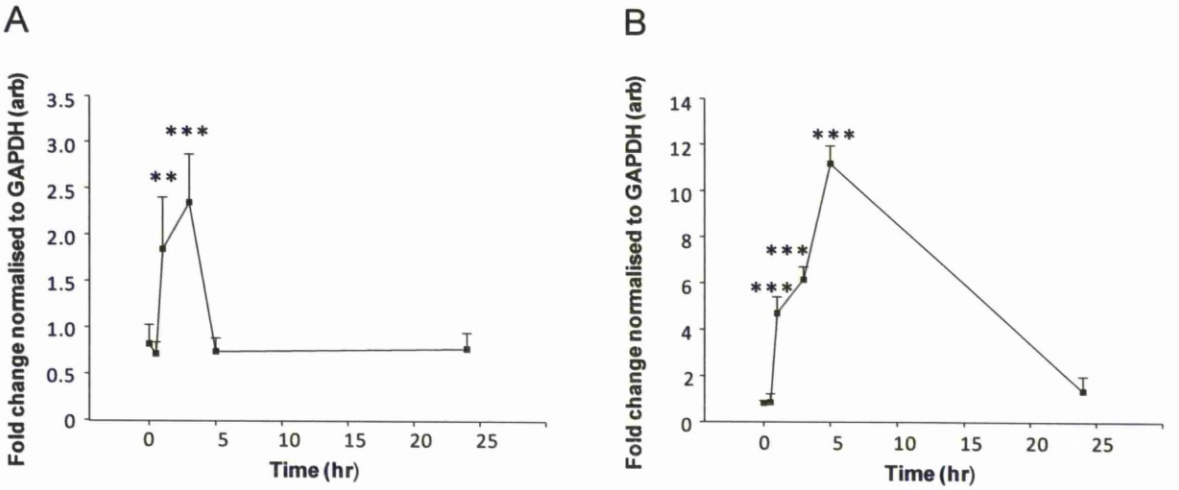

Figure 5.4: Endogenous secretory RAGE mRNA expression in response to APAP-induced hepatotoxicity in fed and fasted mice

The level of endogenous secretory RAGE mRNA expression was assessed by RT-PCR. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 0-24hr) or 0.9% saline. Results were normalised to the house-keeping gene, GAPDH and expressed relative to time-matched saline controls, using the comparative Ct method. Data is given as mean ± SD of 4 mice per group. Statistical significance was assigned relative to time-matched controls. $^{**}p < 0.01$, $^{***}p < 0.001$.

5.3.2 Quantification of RAGE protein during APAP-induced hepatotoxicity

The expression of RAGE protein in membrane fractions of whole liver was assessed by western blot analysis. Male C57BL/6 mice were either fed or fasted prior to administration of either APAP (530mg/kg) or saline and examined 0 to 24hr after dosing. On visual inspection of western blot analyses, and densitometric analysis, membrane expression of RAGE protein in APAP-treated livers in both fasted and fed mice appeared the same as that in livers from saline-treated controls at 0hr (Figure 5.5A and 5.5B). Similar to the findings at 0hr, RAGE protein expression in APAP-treated livers of both fed and fasted mice appeared the same as that in the livers of the respective saline treated controls at 0.5hr (Figure 5.6A and 5.6B), 1hr (Figure 5.7A and 5.7B), 3hr (Figure 5.8A and 5.8B), 5hr (Figure 5.9A and 5.9B) and 24hr (Figure 5.10A and 5.10B). There appeared to be no significant change in level of RAGE protein expression as assessed by visual inspection and densitometric analysis of western blot analyses.

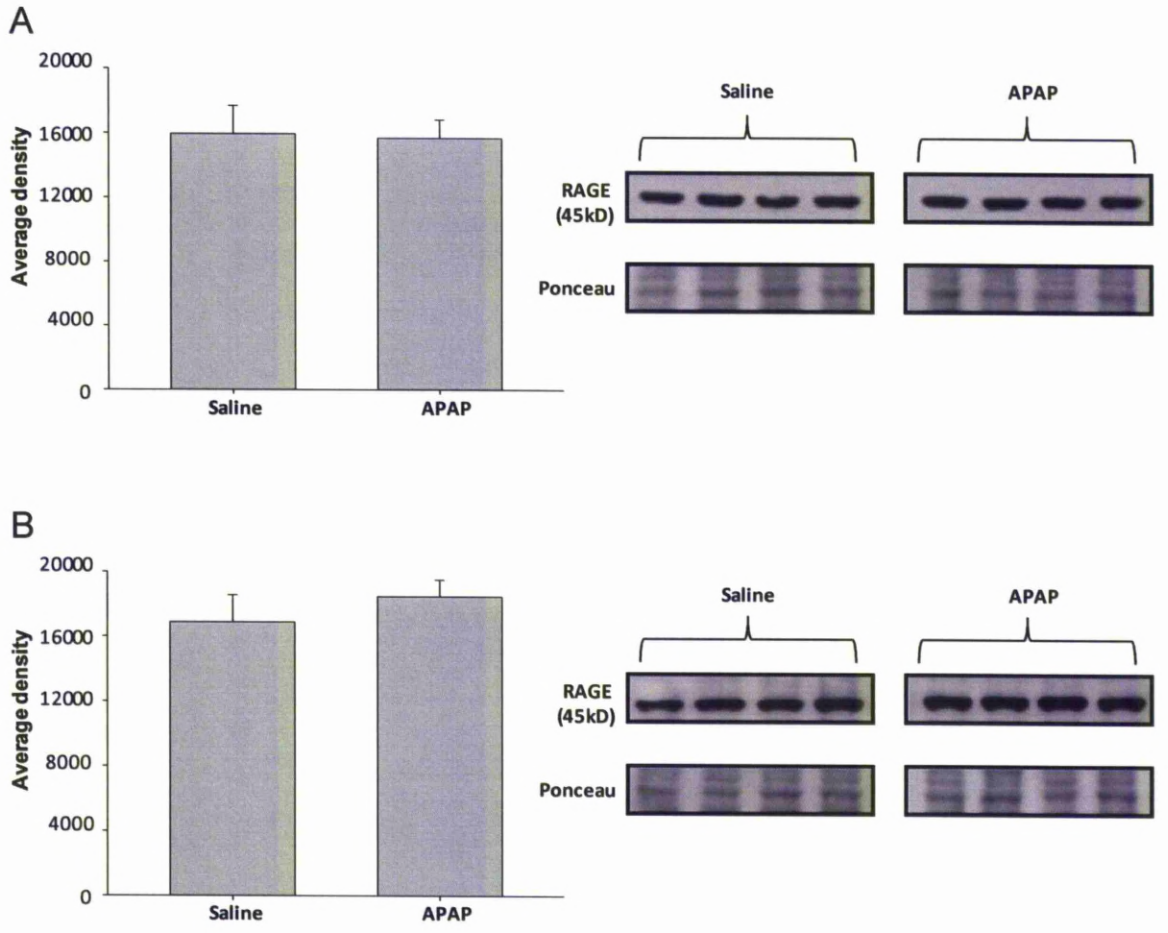

Figure 5.5: RAGE protein expression at 0hr during APAP-induced hepatotoxicity in fed and fasted mice

The level of RAGE protein expression was assessed by western blot analysis. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 0hr) or 0.9% saline. Liver membrane extracts were prepared from frozen liver extracts. Ponceau stains were carried out to ensure equal loading. Data is given as mean ± SD of from densitometric analysis of individual animals (n=4). Statistical significance was assigned relative to respective time-matched control.

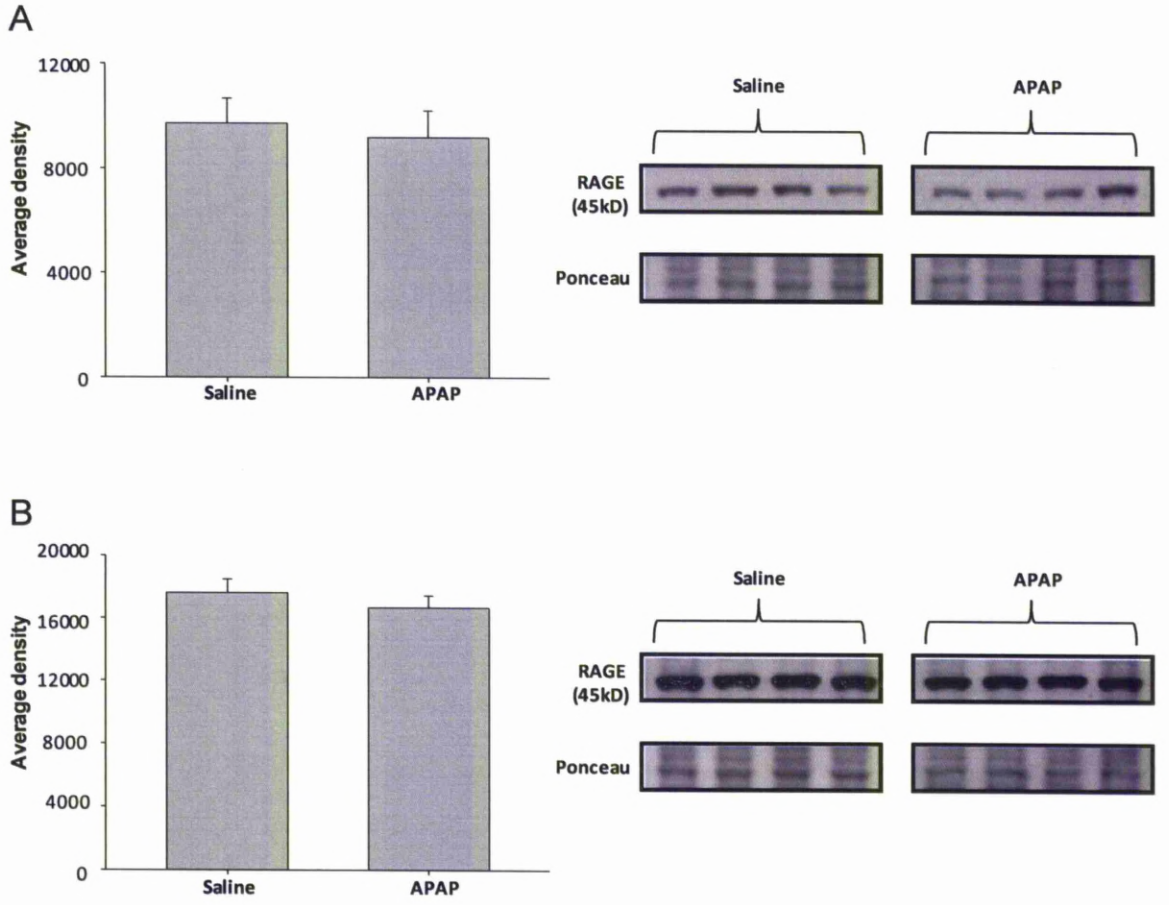

Figure 5.6: RAGE protein expression at 0.5hr during APAP-induced hepatotoxicity in fed and fasted mice

The level of RAGE protein expression was assessed by western blot analysis. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 0.5hr) or 0.9% saline. Liver membrane extracts were prepared from frozen liver extracts. Ponceau stains were carried out to ensure equal loading. Data is given as mean ± SD of from densitometric analysis of individual animals (n=4). Statistical significance was assigned relative to respective time-matched control.

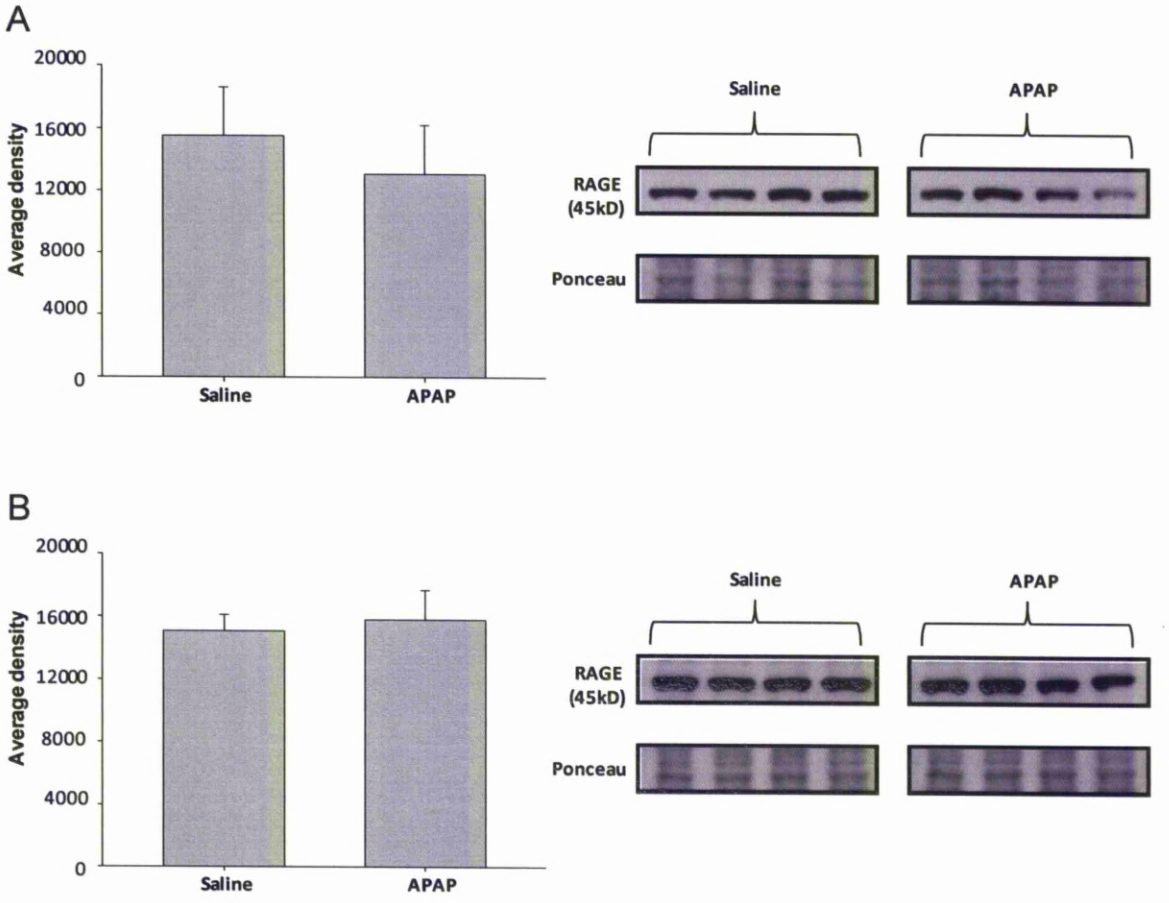

Figure 5.7: RAGE protein expression at 1hr during APAP-induced hepatotoxicity in fed and fasted mice

The level of RAGE protein expression was assessed by western blot analysis. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 1hr) or 0.9% saline. Liver membrane extracts were prepared from frozen liver extracts. Ponceau stains were carried out to ensure equal loading. Data is given as mean ± SD of from densitometric analysis of individual animals (n=4). Statistical significance was assigned relative to respective time-matched control.

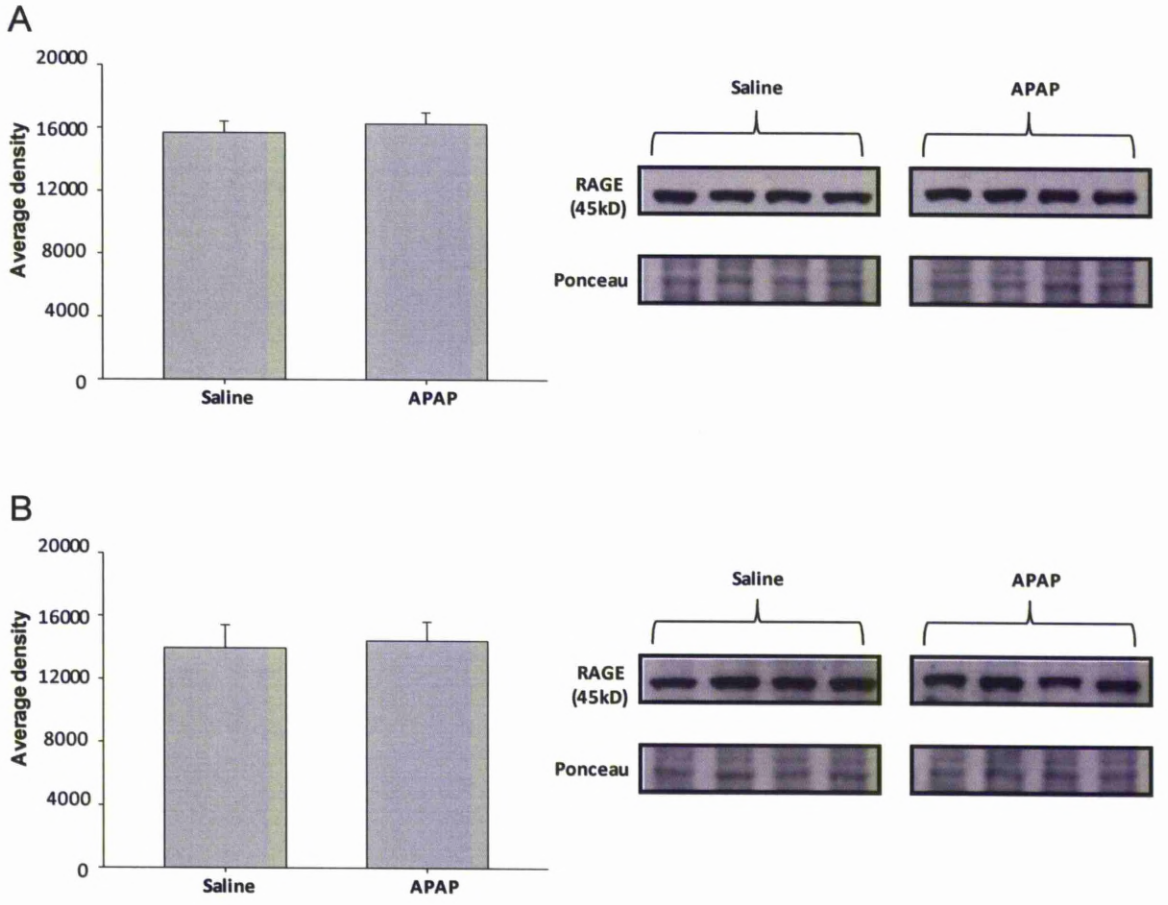

Figure 5.8: RAGE protein expression at 3hr during APAP-induced hepatotoxicity in fed and fasted mice

The level of RAGE protein expression was assessed by western blot analysis. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 3hr) or 0.9% saline. Liver membrane extracts were prepared from frozen liver extracts. Ponceau stains were carried out to ensure equal loading. Data is given as mean ± SD of from densitometric analysis of individual animals (n=4). Statistical significance was assigned relative to respective time-matched control.

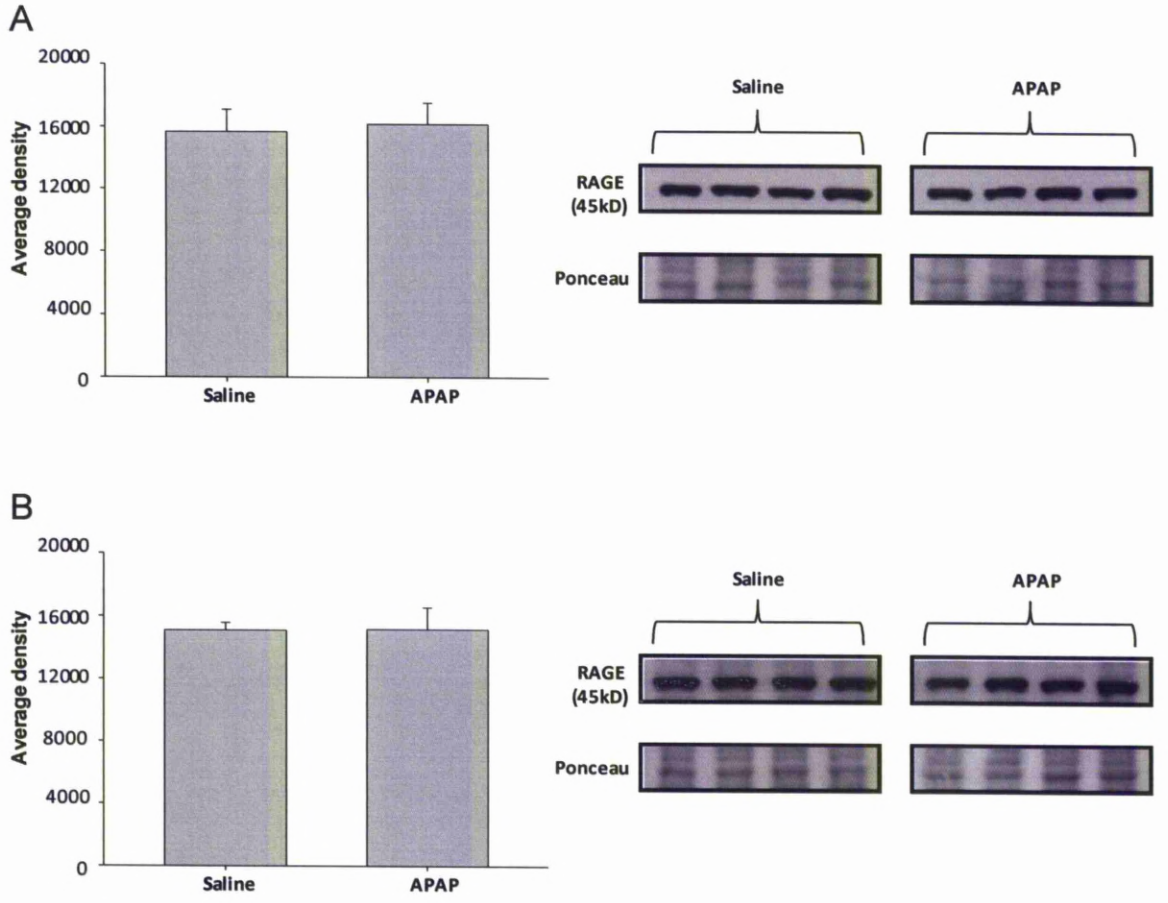

Figure 5.9: RAGE protein expression at 5hr during APAP-induced hepatotoxicity in fed and fasted mice

The level of RAGE protein expression was assessed by western blot analysis. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 5hr) or 0.9% saline. Liver membrane extracts were prepared from frozen liver extracts. Ponceau stains were carried out to ensure equal loading. Data is given as mean ± SD of from densitometric analysis of individual animals (n=4). Statistical significance was assigned relative to respective time-matched control.

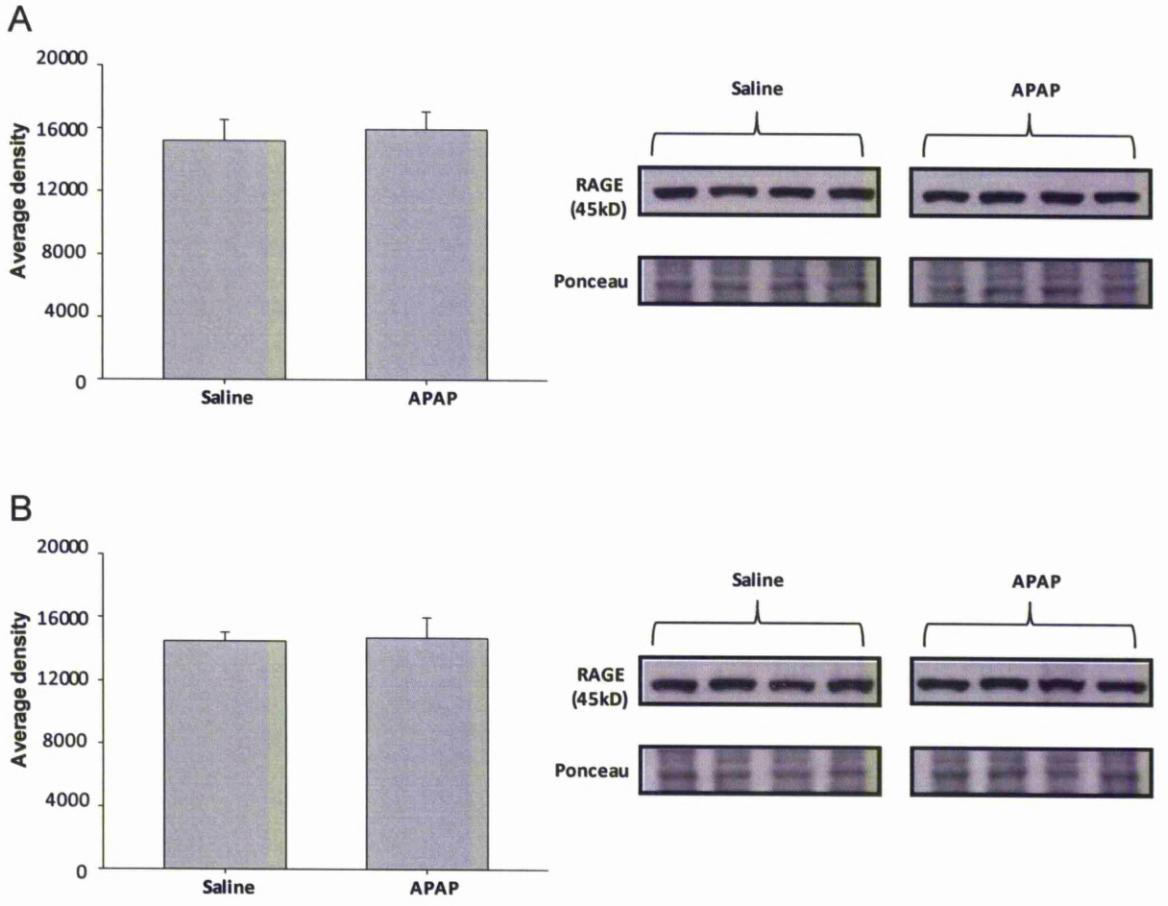

Figure 5.10: RAGE protein expression at 24hr during APAP-induced hepatotoxicity in fed and fasted mice

The level of RAGE protein expression was assessed by western blot analysis. Male C57BL/6 mice were either fed (A) or fasted (B) prior to administration of APAP (530mg/kg; 24hr) or 0.9% saline. Liver membrane extracts were prepared from frozen liver extracts. Ponceau stains were carried out to ensure equal loading. Data is given as mean ± SD of from densitometric analysis of individual animals (n=4). Statistical significance was assigned relative to respective time-matched control.

5.3.3 Cellular expression and localisation of RAGE during APAP-induced hepatotoxicity

To address the cellular localisation of RAGE in the liver of APAP-treated mice, immunohistology and in RNA-situ hybridisation (RNA-ISH) were performed. The histological examination showed the typical APAP-associated centrilobular cell loss at 24hr post-APAP (530mg/kg). RAGE protein expression was seen in the cytoplasm of hepatocytes where it was generally weak, with strongest expression in hepatocytes in periportal areas. RAGE expression was patchy but appeared more intense in remaining cells in areas of centrilobular cell loss (Figure 5.11). This was often associated with a weak nuclear reaction in centrilobular apoptotic hepatocytes (Figure 5.11B). RAGE mRNA expression was represented by a nuclear/cytoplasmic signal in centrilobular apoptotic hepatocytes and in occasional endothelial cells (Figure 5.12). In animals at 3hr post APAP treatment, the RAGE protein and mRNA expression pattern and intensity was similar. In comparison, control livers at 24hr showed constitutive RAGE expression, represented by a variably intense, moderately granular cytoplasmic reaction in a large number of hepatocytes (Not shown). ISH revealed patchy and random distribution of RAGE mRNA, mainly around portal areas and in bile duct epithelial cells (Not shown).

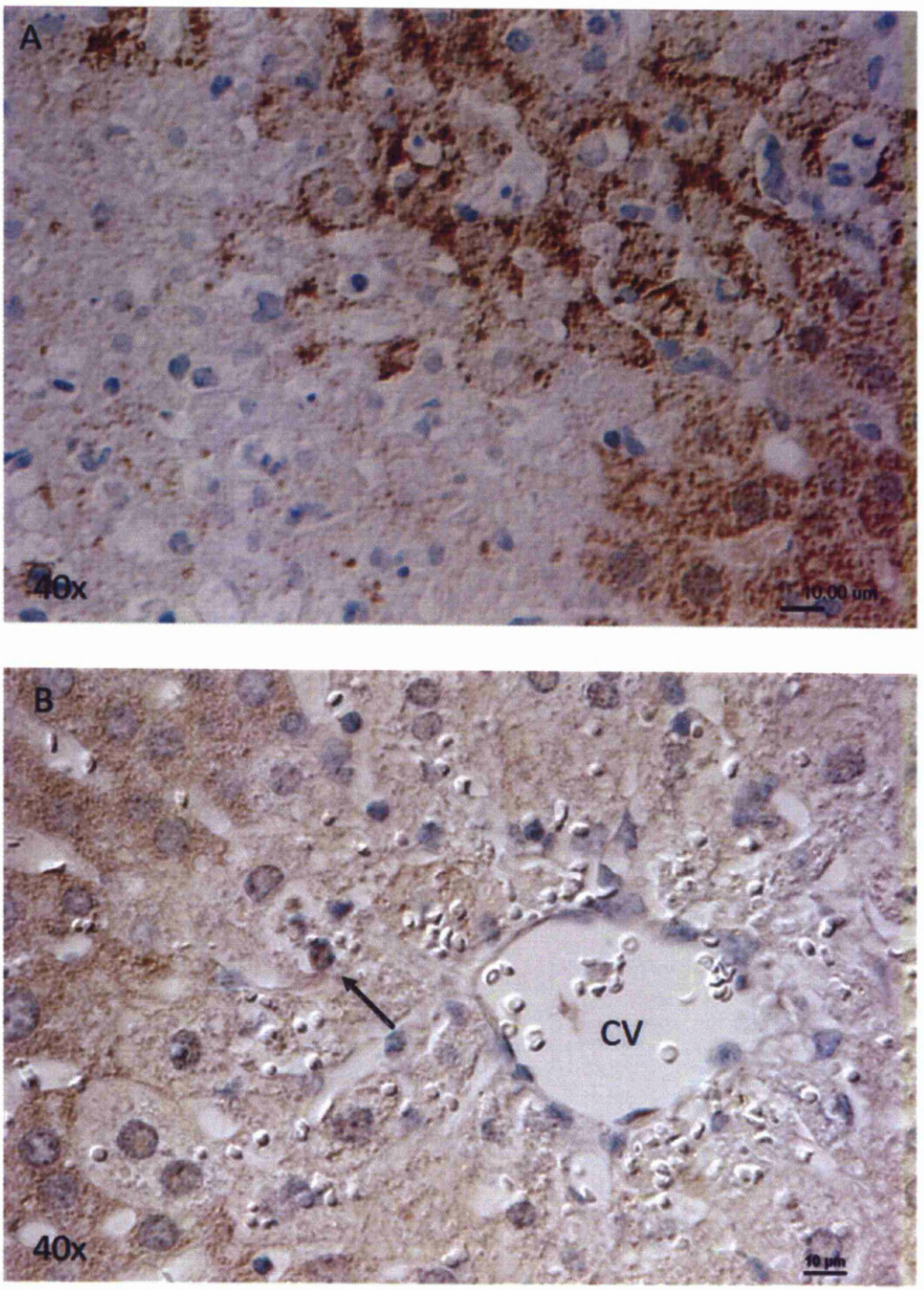

Figure 5.11: Immunohistological demonstration of RAGE protein expression at 24hr post APAP treatment

Male C57BL/6 mice were administered APAP (530mg/kg) and examined 24hr post dosing. RAGE expression is represented by finely granular to patchy cytoplasmic brown staining. A. Most intense expression is seen in intact hepatocytes surrounding areas of centrilobular cell loss. B. Apoptotic hepatocytes exhibit weak nuclear RAGE expression (arrow). CV: central vein.

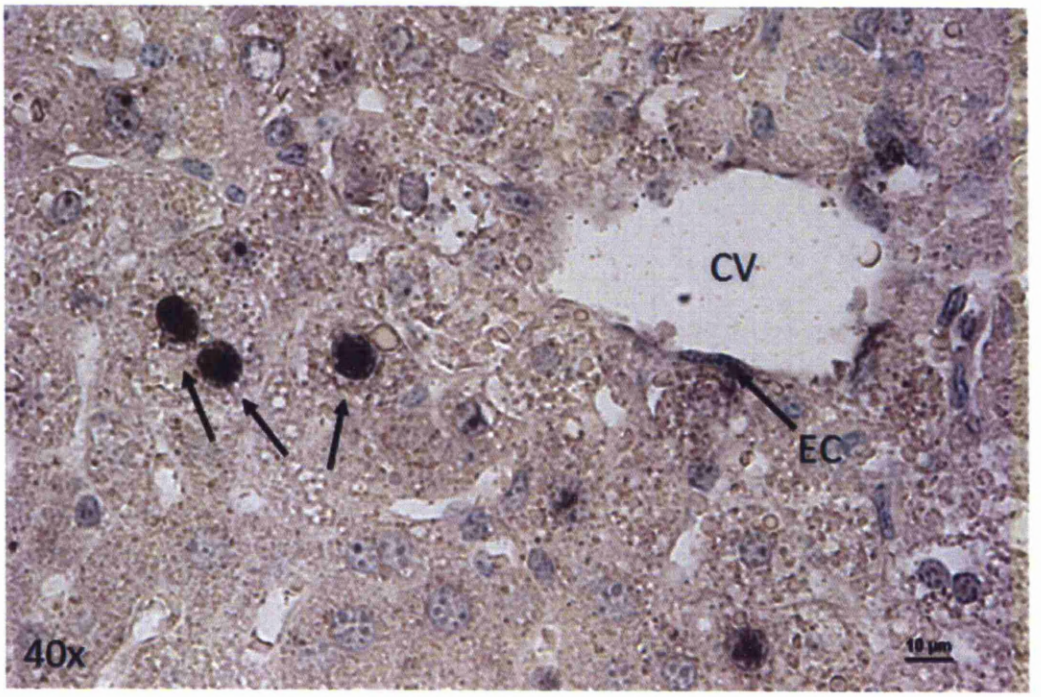

Figure 5.12: RNA *in situ*-hybridisation for RAGE mRNA expression at 24hr post APAP treatment

Male C57BL/6 mice were administered APAP (530mg/kg) and examined 24hr post treatment. The RAGE signal is represented by a dark purple to black nuclear and cytoplasmic staining. Scattered individual hepatocytes (arrows) and scattered endothelial cells (arrow, EC) are positive. CV: central vein.

5.4 DISCUSSION

RAGE is commonly associated and often cited as a molecule that can mediate and exacerbate inflammatory conditions. It is well known that in several pathological states such as diabetes, atherosclerosis and neurodegenerative diseases, RAGE expression is elevated in response to the increased level of its newly formed ligands such as AGEs, S100/calgranulins, amyloid-β-peptides and HMGB1 (Hofmann et al., 1999; Schmidt et al., 2001). As HMGB1 is released by necrotic cells during APAP hepatotoxicity, we reasoned that RAGE may be upregulated in the presence of this ligand. The aims of the experiments presented in this chapter was to provide evidence for the possible participation of RAGE in APAP-induced liver injury by analysis of hepatic expression of RAGE at the mRNA, protein and tissue level in our established model of APAP hepatotoxicity and inflammation.

RAGE is expressed both constitutively and inducibly in a wide variety of cell types such as vascular smooth muscle cells, neurons, lung alveolar epithelial cells, endothelial cells and mononuclear phagocytes (Brett et al., 1993; Katsuoka et al., 1997). However, except for mouse lung tissue, where RAGE is abundant, a number of reports indicate that RAGE mRNA and protein are expressed at low or barely detectable levels respectively, in a variety of different cell types such as monocytes/macrophages, endothelial cells, smooth muscle cells, fibroblasts and neuronal cells (Brett et al., 1993; Bierhaus et al., 2005; Demling et al., 2006; Raucci et al., 2008). While basal levels of RAGE are seen to be low, these cells can be induced to express RAGE in conditions in which inflammatory mediators and ligands accumulate (Huttunen et al., 1999). Despite its very low expression level, the ubiquitously expressed RAGE has been reported to mediate HMGB1 signalling responses by a number of cell types, including macrophages, myoblasts and neutrophils (Kokkola et al., 2005; Riuzzi et al., 2006; Muhammad et al., 2008; van Zoelen et al., 2009). RAGE has been seen to increase in the presence of HMGB1 in mouse embryonic fibroblast cells (Penzo et al., 2010). Also, a recent study showed that macrophage expression of RAGE mRNA is upregulated in response to conditioned medium containing HMGB1, collected 24hr after treatment of mouse hepatocytes with APAP (Dragomir et al., 2011). To the best of our knowledge, there have been no reports that determine the expression of RAGE *in vivo* in situations such as APAP-induced liver injury.

RAGE mRNA expression was compared in our fasted and fed APAP model over a time course of APAP treatment (530mg/kg) ranging from 0-24hr, similar to the APAP time course studies performed in Chapter 2. The comparison of the dietary state of mice was employed as the findings in Chapter 2 showed that fasted mice were more susceptible to the toxic effects of APAP due to increased bioactivation and subsequently invoked a greater inflammatory response (Figure 2.11 and 2.12 respectively). It was hypothesised that the greater the inflammatory response, the more likely it would be that RAGE would be induced and subsequently expressed. Real-time PCR experiments were performed with a set of RAGE PCR primers that would detect all RAGE mRNA isoforms (total RAGE), full-length RAGE and endogenous secretory RAGE in whole liver preparations of mice. Total RAGE mRNA expression was upregulated in fed mice in response to APAP treatment compared to saline treated controls at 3hr. The time points before and after 3hr showed no upregulation and remained at basal levels. However, in fasted mice, total RAGE mRNA upregulation occurred at an earlier time point of 1hr and continued to increase until 5hr, suggesting a more prolonged induction (Figure 5.2). Next, the expression of the full-length RAGE isoform was assessed. flRAGE is bound to the cell membrane and contains the cytosolic domain that appears essential for intracellular signalling (Huttunen et al., 1999). flRAGE mRNA expression in fed animals exhibited a similar trend to that observed with total RAGE in that upregulation occurred at only the 3hr time point. Fasted animals also continued the trend in which upregulation of flRAGE occurred earlier at 1hr and was sustained until 5hr where the expression levels had peaked (Figure 5.3). Finally the expression of endogenous secretory RAGE mRNA was determined. esRAGE is a variant that encodes a secreted form lacking the transmembrane and cytosolic domains, which appears to function as a decoy receptor that neutralises circulating ligands. Upregulation of esRAGE mRNA expression in fed mice occurred at 1hr, earlier than that observed with total and full-length RAGE, and continued to increase at 3hr. After 3hr expression went back to basal levels. In fasted mice, the familiar trend of total and full-length RAGE was observed with expression increasing at 1hr and continuing to increase until 5hr, in which peak upregulation occurred (Figure 5.4).

The time course dynamics of each of the RAGE isoforms in response to APAP may be explained when comparing alongside the toxicity and inflammation dynamics established in the APAP model in Chapter 2. In fed animals, toxicity occurred at 3hr post APAP-dose along with a trend of increasing serum inflammatory cytokines levels (Figure 2.9 and 2.10). At this

point upregulation of total RAGE, flRAGE and esRAGE occurred. However, toxicity and cytokine levels persisted at both 5hr and 24hr whereas RAGE mRNA expression in all three forms had returned to basal levels. In fasted mice, toxicity also occurred at 3hr but at a greater level than in fed (Figure 2.11). Similarly, serum levels of cytokines increased at this time point and were also at a greater level in fasted animals than in fed (Figure 2.12). The level of expression of RAGE mRNA exhibited the same trend as the fold increase of each RAGE form was much greater in fasted animals than in fed at the equivalent time points. These results suggest that RAGE mRNA is upregulated to a greater level in more pronounced toxic and inflammatory conditions, however the physiological or pathological role of RAGE in APAP-induced liver injury may be mediated through complex interplay between the RAGE isoforms, and may not be mediated solely by full-length RAGE. flRAGE was reported to be the most predominant form of RAGE (Kalea et al., 2009) and is responsible for producing sustained cellular activation. The presence of more flRAGE presumably results in greater and more sustained signalling through the flRAGE receptor. In this scenario, esRAGE may be upregulated in response to APAP toxicity to act as a negative feedback mechanism, to stop ligands interacting with full-length RAGE and therefore suppressing overexpression and continuous perpetuation of inflammatory signalling.

In contrast to the mRNA data, RAGE showed a different protein expression pattern as shown by western blot analysis in isolated membrane fractions of livers in APAP-treated mice. At each of the time points post APAP-dose there was no upregulation or change in expression levels of RAGE protein. Basal levels of RAGE protein could be detected in control animals at each time point and there was no difference between them and time-matched APAP-treated groups. The full extent of RAGE expression has yet to be fully deciphered. It is known however, that the full-length form of RAGE may be proteolytically cleaved proximal to the membrane to generate a soluble form of RAGE (sRAGE) that will have the same decoy capacity as esRAGE (Raucci et al., 2008). Ectodomain cleavage, also known as 'shedding' regulates the functions of many membrane-bound proteins (Schlondorff et al., 1999; Huovila et al., 2005; Garton et al., 2006; Reiss et al., 2006). The proteases responsible for shedding belong to the family of metalloproteases, in particular, the ADAM (a disintigrin and metalloproteinases) family. Generally, shedding occurs constitutively but can be induced by ligand binding (Raucci et al., 2008). Moreover, binding of HMGB1 has been reported to promote RAGE shedding (Raucci et al., 2008). The RAGE antibody used in this investigation

detected full-length RAGE only, with no evidence for cross-reactivity to other forms of RAGE as the western blot data showed only one band with a molecular weight of 45kD. Further investigation would be required to monitor sRAGE levels in the serum, as sRAGE may be shed during APAP hepatotoxicity and thereby would not be detected in membrane fractions of the liver using a flRAGE specific antibody. It has also been demonstrated that RAGE can be internalised by engagement of its ligands. A study by Sevillano et al. (2009) showed that binding of RAGE with its ligand AGEs led to internalisation and that this process was needed for the generation of intracellular responses mediated by AGEs. So far it is unknown if internalisation of RAGE via HMGB1 is required to generate an intracellular response but evidence exists that RAGE is internalised with HMGB1-DNA complexes (Tian et al., 2007). Internalisation of membrane-bound RAGE may prevent its detection by western blot analysis, however if RAGE is concurrently being upregulated then any change in the level of surface RAGE may not be detected. Together, these results could be a valid reason why there was no observable difference in RAGE protein expression and why there was no correlation between mRNA and protein expression. HMGB1 alone may promote the increase and upregulation of RAGE levels by direct interaction but may also help maintain the balance of cell surface RAGE levels simultaneously subject to rapid depletion by HMGB1 engagement resulting in RAGE shedding and internalisation. The physiological and/or pathological factors that regulate shedding and internalisation of flRAGE *in vivo* require further examination.

Both immunohistological and *in situ* detection of RAGE protein and mRNA confirmed constitutive RAGE production in the liver, which was mainly seen in hepatocytes. Expression was highly variable and often patchy, but also appeared random in control mouse livers. There was evidence of upregulation in degenerating cells post APAP treatment. An interesting finding seen with immunohistology was that RAGE protein was found to be localised in the nucleus of apoptotic hepatocytes post APAP treatment. This finding requires further investigation to determine if this is a functional response by cells to sequester RAGE in the nucleus of hepatocytes following APAP hepatotoxicity to prevent further inflammatory signalling, thereby acting as a negative feedback loop to prevent prolonged inflammation.

The contribution of RAGE activation to APAP hepatotoxicity has been touched upon previously in one study (Ekong et al., 2006). The authors found that blockade of RAGE using sRAGE led to increased survival and a reduction in oxidative stress in APAP-treated animals. However, the full extent of RAGE expression and the molecular mechanisms that control it have not been evaluated adequately. Gaining further knowledge about the molecular mechanisms underlying RAGE and esRAGE regulation may provide new diagnostic and therapeutic options to pursue the reduction of RAGE-mediated exacerbation of APAP-induced liver injury. Continuation of this work to elucidate the contribution of RAGE in APAP toxicity, such as use of $RAGE^{-/-}$ mice and antibodies and pharmacological inhibitors targeted at neutralising RAGE may provide a clearer picture and a novel approach to reducing APAP hepatotoxicity.

CHAPTER SIX

CONCLUDING DISCUSSION

CONTENTS

6.1 Background

Drug-induced liver injury (DILI) has proven to be a major problem for both the pharmaceutical industry and regulatory authorities and is the most frequent cause for withdrawal of approved drugs and attrition of drug development (Lazarou et al., 1998; Jaeschke et al., 2002; Pirmohamed et al., 2004). The key to predicting and preventing DILI is by having a thorough understanding of all the underlying mechanisms. The overall aim of the work presented within this thesis was to explore the mechanism of inflammation associated with DILI with the ultimate aim of applying these findings to further understand the various inflammatory pathways during DILI and their contribution to the progression of liver injury. Figure 6.1 shows a general overview of the mechanisms of inflammation associated with DILI, highlighting the intracellular mechanisms that have been explored in this thesis. These were investigated by;

- Developing suitable models of DILI and inflammation. The characterisation of these animal models in Chapter 2 enabled their use in further studies presented in chapters 3, 4 and 5 with the aim to assess the effect of modulating certain parts of the inflammatory pathway associated with DILI such as leukocyte infiltration and signalling (Chapter 3), signalling by dying hepatocytes and immune cells (Chapter 4) and the receptors associated with these danger signals (Chapter 5). Collectively, the models provided useful experimental systems for the investigation of the underlying mechanisms of DILI-associated inflammation. Also, the effect of fasting on APAP-hepatotoxicity and inflammation was addressed.

- Evaluating the contribution of the innate immune response in the pathogenesis of APAP-induced liver injury. The APAP model was employed in Chapter 3 to identify the role DMSO played in modulation of leukocyte accumulation as well as using aspirin as a pharmacological inhibitor of the Nalp3 inflammasome in our models of hepatotoxicity and inflammation that were established in Chapter 2. This work served to assess the potential of aspirin as a modulator of the inflammatory response in our models by its reported effect on the Nalp3 inflammasome in neutrophils.

- Exploring the downstream signalling events triggered by DILI and by LPS exposure. The work conducted in Chapter 4 investigated the potential protective effect of inhibiting early (TNF-α, IL-6 and IL-1β) and late (HMGB1) inflammatory cytokines in our APAP and LPS models. This was investigated using ethyl pyruvate (EP) as a pharmacological inhibitor of these cytokines and the outcome was compared to the effects of complete HMGB1 inhibition by the use of an anti-HMGB1 antibody.

- Evaluating the expression of the HMGB1 receptor RAGE in the tissues of APAP-treated mice. The work in Chapter 5 was conducted with the intent to deduce its relationship to the pathophysiology of APAP hepatotoxicity as the expression of RAGE was investigated in our APAP model at the mRNA, protein and tissue level using RT-PCR, Western blot analysis, immunohistology and in situ hybridisation.

The work undertaken was to assess the contribution of inflammation towards DILI by targeting certain points in the signalling pathway between dying hepatocytes and cells of the innate immune system. Further investigation of this mechanism of inflammation associated with DILI will aid the development of effective methods of therapeutic intervention and assessment of novel mechanisms by understanding the relationship between inflammation and toxic outcome.

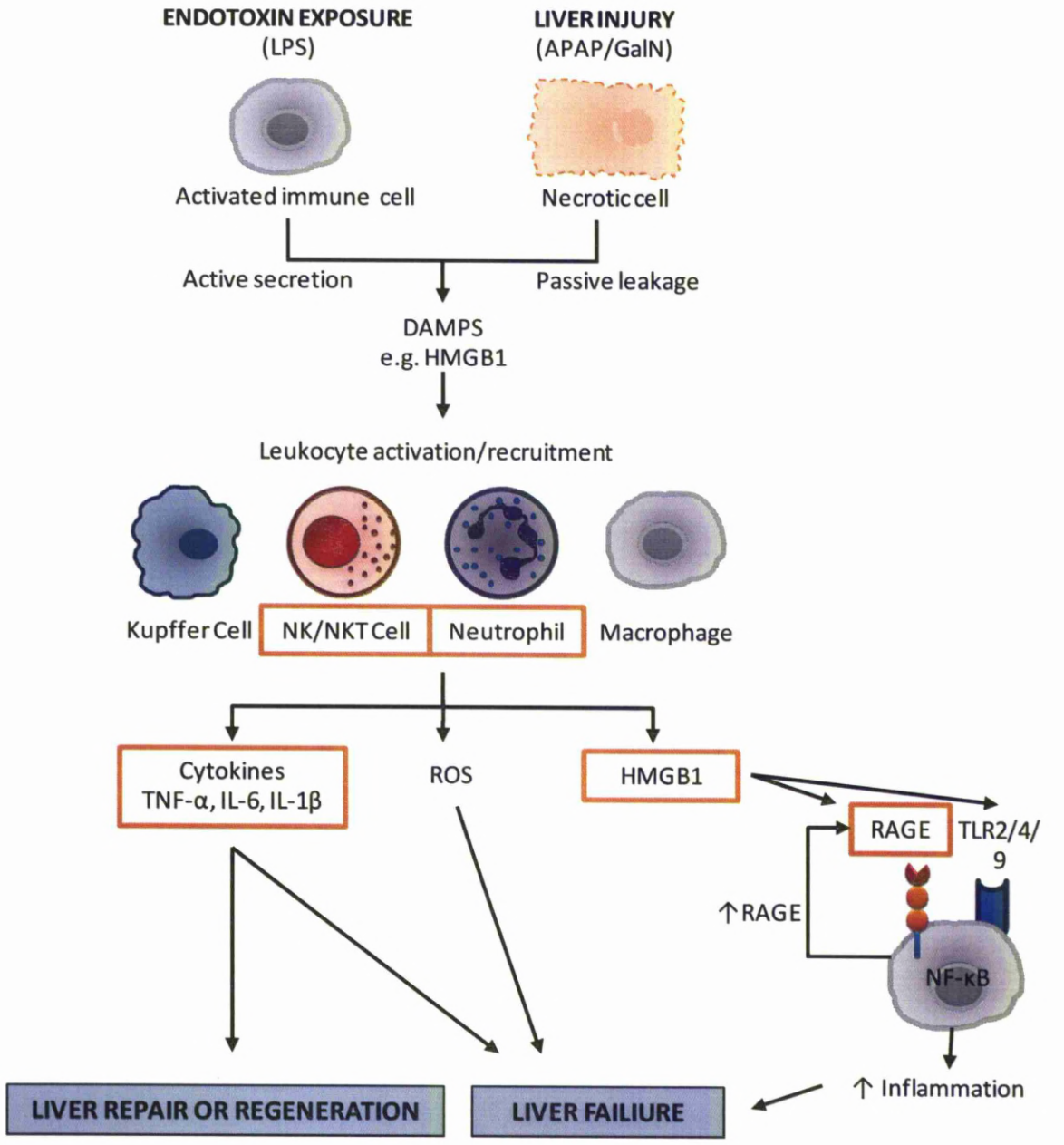

Figure 6.1: Schematic overview of the inflammatory pathway during DILI and sterile inflammation.

Drugs or chemicals cause cell injury and necrosis, which results in the release of cellular contents including DNA, RNA, and proteins (HMGB1). These DAMPs, can bind to innate immune cells, triggering cytokine and chemokine production. Innate immune cells can then be activated and/or recruited to the liver where they produce pro- and anti-inflammatory mediators, ROS and acetylated HMGB1. Signalling of HMGB1 through receptors RAGE and TLRs can lead to prolonged inflammation and subsequent liver damage. Signalling through RAGE can lead to a positive feedback loop in which the RAGE signal is maintained and amplified. Orange highlighted boxes represent mechanisms and factors that have been explored in this thesis either by modulation or measurement.

6.2 Animal models of DILI-associated inflammation

Currently, sufficient data to support an unequivocal role for inflammation in DILI for drugs are lacking, and mechanistic details are unknown. Evidence to support the hypothesis that DILI triggers the activation of the innate immune system within the liver has been obtained from experimental *in vivo* models. The advantages of investigating inflammation in animal models of DILI are the stable genetic, molecular and immunological backgrounds of inbred strains. This lack of variation, compared to the clinical situation, enables a more complete exploration and determination of possible inflammatory mechanisms contributing to the overall pathophysiology downstream from drug metabolism or accumulation. Several different models of DILI in which inflammation has been modulated have been investigated in an attempt to dissect the processes of inflammation from those of direct drug-induced hepatocyte cell death. However, the use of different strains of mice, species, compounds and the varying doses and pretreatments in *in vivo* models in the literature, have led to conflicting reports regarding the role that the inflammatory response plays in the pathogenesis of DILI, most notably, in APAP-induced liver injury.

The initial studies presented within this thesis were designed to define a set of *in vivo* models of DILI that could produce an inflammatory response which could be assessed and characterised by a chosen set of observable or measurable parameters. The use of these models in this thesis allowed for the investigation of various inflammatory pathways during DILI and by modulating these pathways, their contribution to the progression of liver injury could be examined. Primarily the development of an APAP model that could consistently provide reproducible levels of toxicity and inflammation was the main focus, so that further studies involving modulation of inflammation could be compared back to the original model as well as comparisons to other models of hepatotoxicity and inflammation with different mechanistic pathways. LPS was used as a systemic innate immune system activating agent, to produce a potent inflammatory response without causing hepatic cell damage, while two model hepatotoxins, APAP and GalN were utilised to produce combinations of toxicity and an inflammatory response. Both APAP and GalN provide a toxicological endpoint in an integrated animal model and require metabolic activation to initiate toxicity, however, there is a biochemical difference in the initiation of hepatotoxicity between APAP and GalN as APAP hepatotoxicity requires GSH depletion but GalN toxicity does not (Mitchell et al.,

1973b; Decker and Keppler, 1974). High doses of GalN are known to cause hepatic necrosis by UTP depletion, leading to inhibition of RNA synthesis (Decker and Keppler, 1974). GalN was chosen as a model hepatotoxin as it has been frequently used to induce experimental liver injury (Keppler et al., 1968), has a different mechanism of toxicity to APAP and has been shown to produce an inflammatory response (Strachlewitz et al., 1999). Using these different agents was aimed at generating multiple models so we could characterise the type of immune and inflammatory activation that APAP caused and then compare with both LPS and GalN. This would give mechanistic data to help understand the nature of the immune response to APAP. APAP-induced liver injury is a widely studied model of DILI in mice and has been extensively used to investigate the involvement of inflammation. Table 6.1 shows a general overview of the inflammatory models used in APAP hepatotoxicity and highlights just how many different models have been used to determine the role of inflammation with APAP. The use of different doses of APAP, along with different strains and genetic backgrounds of mice has made it difficult to fully define an inflammatory response or the subsequent role it plays in DILI. Once validated, the APAP model used in this thesis produced relatively consistent levels of toxicity and inflammation, reducing variability between studies. Also, in an attempt to further reduce variability the C57BL/6 mouse strain was chosen due to its inbred background and common use in the literature for APAP, LPS and GalN investigations (Bahrami et al., 1994; Nowak et al., 2000; Masubuchi et al., 2003; Liu et al., 2004; Liu et al., 2006; Bourdi et al., 2007; Maddox et al., 2010; Zurita et al., 2010).

The comparison of the toxicological response to APAP and GalN is important as both compounds initiate toxicity via different mechanisms, as mentioned previously, and this was reflected by the level of inflammation induced by each. Both compounds were used as tools to induce a specific response. The results generated by dose response studies in Chapter 2 has shown that APAP and GalN induced dissimilar levels of a toxicological response and that LPS caused no toxicity, but all three agents resulted in an inflammatory response. The GalN model was characterised by mild hepatocellular death and moderate inflammation whereas APAP produced a more severe pattern of hepatocellular death and a systemic inflammatory response. LPS, despite apparent lack of toxicity, not inducing hepatocyte cell death induced a potent systemic inflammatory response as characterised by cytokine release into the serum. This supports findings in the literature that no hepatotoxicity was observed in mice that received LPS alone (Maddox et al., 2010). Administration of LPS induces systemic

inflammation characterised by the production of a range of cytokines (Wong et al., 2000). LPS has been used more as an adjuvant to DILI in recent studies, as it has been shown that mice treated with an inflammatory dose of LPS became more sensitive to APAP-induced liver injury. LPS co-administration caused a leftward shift in the dose-response curve for APAP hepatotoxicity, causing normally nontoxic doses of APAP to become hepatotoxic (Maddox et al., 2010). The inflammation produced in this model is likely to have been by activated immune cells rather than the release of inflammatory contents by damaged or dying hepatocytes. Having an inflammatory model based predominantly on activation of the innate immune system as opposed to cell damage enabled the investigation of the mechanism of inflammation by means of modulating the inflammatory pathway and apportioning the level of toxicity associated caused by inflammation or the direct deleterious effect of the model hepatotoxins.

The conflicting reports in the literature regarding the role of the inflammatory response induced by APAP could also be a consequence of fasting experimental animals. Previous literature reports using histology noticed that a common feature in mice fasted prior to investigation of the APAP-induced inflammatory response was the inability of these mice to recover from APAP damage at 24hr (Bourdi et al., 2002; Liu et al., 2006). Moreover, within the investigations in Chapter 2, fasted animals responded differently to non-fasted animals post APAP with a much greater toxicological and inflammatory response observed, suggesting that fasting caused animals to be more susceptible to toxic agents and subsequently more inclined to induce a greater inflammatory response. Fasting of animals can profoundly affect the toxicological response by decreasing hepatic GSH (Shimizu et al., 1992), and ATP (Lee et al., 1988), altering CYP450 expression (Hu et al., 1995) and downregulating gene expression associated with apoptosis (Bauer et al., 2004). Further investigations are required to fully determine why fasting prior to experimental DILI elicits a greater inflammatory response in animals. One hypothesis is that fasting causes a shift towards necrosis as the predominant mode of cell death as fasting leads to depletion of intracellular ATP, inhibiting the energy-dependent process of apoptosis (Kim et al., 2003). Increasing the level of necrosis would lead to the release of more inflammatory signals and mediators, promoting a greater inflammatory response. By considering the dissimilar toxicological and inflammatory responses to APAP in fasted and fed mice, the results in Chapter 2 highlight important implications for the planning and execution of mechanistic

inflammatory studies in *in vivo* models. Work carried out previously by Antoine et al. (2010) supports the current finding in this thesis that fasting promotes the induction of inflammation by a more severe toxicological response. This is advantageous in models designed to evaluate and measure inflammatory outputs, however, caution is required when interpreting results in fasted animals when studying biological and physiological mechanisms leading to cell death and organ failure. This is further highlighted when considering that the ultimate goal of animal models is the prediction of human DILI during drug development (Dixit and Boelsterli, 2007). A combination of factors in the literature, such as fasting mice, using different doses of APAP and using different strains and genetic backgrounds of mice could offer an explanation for the controversy and inconsistency in reports for the role of inflammation in APAP-induced liver injury. Therefore the initial aim in this thesis was to produce a set of *in vivo* models that could aid further investigations involving modulation of inflammatory pathways, including a consistent and reproducible model of APAP-induced liver injury and inflammation.

Table 6.1: Summary of inflammatory models used in APAP-induced hepatotoxicity.

References	Inflammatory model(s)	Reported effect
Ju et al., 2002; Goldin et al., 1996	KC depletion	Protection against liver injury at early time points, more susceptible at later time points
Liu et al., 2006	Neutrophil depletion	Significantly protected against liver injury
Liu et al., 2004	NK/NKT cell depletion	Significantly protected against liver injury
Blazka et al., 1995	Anti-TNF-α/IL-1-α/IL-1ra	Increased susceptibility to liver injury
Ishida et al., 2004	Anti-TNF-α,TNF-p55 receptor $1^{-/-}$ mice	Reduced mortality and liver injury
Chiu et al., 2003	$TNFR1^{-/-}$ mice	Increased susceptibility to liver injury
Ishida et al., 2002	IFN-$\gamma^{-/-}$ mice	Significantly protected against liver injury
Masubuchi et al., 2003	IL-$6^{-/-}$ mice	Increased susceptibility to liver injury
Bourdi et al. 2007	IL-10/4/$6^{-/-}$ mice	Significantly protected against liver injury
	IL-10/$4^{-/-}$ mice	Increased susceptibility to liver injury
Yee et al., 2007	IL-$13^{-/-}$ mice	Increased susceptibility to liver injury
Imaeda et al., 2009	Anti-TLR9, $TLR9^{-/-}$, $Nalp3^{-/-}$ mice	Reduced mortality and liver injury
Williams et al., 2011	$Nalp3^{-/-}$ mice	No protection against liver injury
Ekong et al., 2006	Soluble RAGE	Reduced mortality and liver injury
Maddox et al., 2010	LPS	Increased susceptibility to liver injury

6.3 Immune cell infiltration and modulation

Growing evidence suggests that the innate immune system can contribute to the severity and progression of DILI for certain drugs through the production of cytokines and recruitment of inflammatory cells into the liver downstream from cell damage (Blazka et al., 1995; Laskin et al., 2003; Ishibe et al., 2009). The controversy regarding the role of the innate immune system in the overall pathogenesis of DILI stems from conflicting literature reports concerning the importance of various leukocyte populations for the progression and exacerbation of APAP induced liver injury (Liu et al., 2006; Masson et al., 2008). It is important to better understand the mechanisms by which cells of the immune system are activated and recruited into the liver during drug-induced hepatotoxicity and how these cells cause hepatocellular injury. One of the aims of this thesis was to modulate the recruitment and signalling of innate immune cells during drug-induced toxicity and assess the relative contribution of these immune cells to liver injury.

The pathological role of NK/NKT cells in a murine model of APAP-induced liver injury was reported to be secreting IFN-γ, modulating chemokine production, accumulation of neutrophils, and up-regulating FasL expression in the liver, all of which may promote the inflammatory response of liver innate immune system (Liu et al., 2004). It was also reported in the same study that depletion of these cells in the liver exerted a protective effect against APAP hepatotoxicity by substantially reducing the formation of cytokines (notably IFN-γ), chemokines and neutrophil recruitment. However, it was later determined that the presence of DMSO as a solvent in these studies activated NK and NKT cells, which does not occur without DMSO (Masson et al., 2008). It was found that DMSO used in these initial studies increased the activation of these NK/NKT cells and that their depletion did not affect APAP hepatotoxicity unless the cells were activated by DMSO prior to APAP. One of the studies carried out in this thesis was designed to evaluate the effect of DMSO on infiltrating immune cells in our APAP animal model and if this affected the progression of APAP hepatotoxicity. The results generated in Chapter 3 showed that the APAP model demonstrated a toxicological response consistent with that seen in Chapter 2. APAP, when administered concurrently with DMSO, showed a complete inhibition of toxicity at 24hr but when DMSO was administered 2hr or later after APAP, the level of toxicity was significantly reduced. Moreover, DMSO demonstrated a dose dependent reduction in APAP toxicity when

administered concurrently. The primary effect of DMSO in our APAP model was inhibition of toxicity, likely due to the inhibition of APAP bioactivation by DMSO as there was no hepatic GSH depletion when a high dose of DMSO (1mg/ml) was administered concurrently with APAP. These findings are in support of literature evidence that report that high levels of DMSO can have an inhibitory effect on CYP450 metabolism and toxicity (Park et al., 1988; Lind et al., 2000; Dunphy et al., 2007), as APAP bioactivation is dependent on CYP450 enzymes (Dahlin et al., 1984; Raucy et al., 1989; Thummel et al., 1993). The study by Masson et al. (2008) did not confirm whether DMSO had an impact on APAP metabolism. Overall, the results presented in Chapter 3 suggest the effect of DMSO on exacerbation of APAP-toxicity by recruitment of inflammatory cells is minimal as the inhibitory effect of DMSO on the enzymatic bioactivation of APAP and subsequent reduction of hepatotoxicity is more prominent when DMSO is administered concurrently with APAP. These results are supported by a previous study in that high doses of DMSO inhibit metabolic activation of APAP, resulting in protection against APAP hepatotoxicity (Jaeschke et al., 2006b). The use of DMSO to facilitate the dissolution of compounds in studies involving the hepatic immune system must be carefully considered, due to the ability of DMSO at high doses to inhibit bioactivation of compounds requiring CYP450 enzymes.

The pathogenic role of neutrophils has been established in cases of liver disease such as ischaemia-reperfusion mediated liver injury (Jaeschke et al., 2003b; Hasegawa et al., 2005), alcoholic hepatitis (Bautista., 1997), obstructive cholestasis (Gujral et al., 2003b) and α-naphthylisothiocyanate (ANIT) toxicity (Hill et al., 1999; Xu et al., 2004). However, the evidence for the involvement in DILI has been limited to a few examples, such as halothane (You et al., 2006), concanavalin A (Bonder et al., 2004) and α-naphthylisothiocyanate (Dahm et al., 1991). Neutrophils have been implicated as having an active role in the pathogenesis of APAP hepatotoxicity in mice (Ishida et al., 2006; Liu et al., 2006), where depletion of neutrophils in mice led to reduced toxicity and improved survival. However, many other studies do not support a relevant contribution of neutrophils to exacerbation of liver injury. Recently, it was implied that neutrophil infiltration, partially mediated by IL-1β, could exacerbate liver injury after APAP (Imaeda et al., 2009). The inflammatory pathway investigated in this study was the Nalp3 inflammasome that regulates activation of caspase-1 that in itself regulates formation of IL-1β, leading to neutrophil recruitment and a subsequent inflammatory response. The investigators reportedly reduced APAP-induced liver injury by

using gene knockout mice of each component of the inflammasome and also aspirin as a pharmacological inhibitor. However, the role of neutrophils in progression of APAP hepatotoxicity has been contested (Bauer et al., 2000; Lawson et al., 2000; Cover et al., 2006). It was suggested that even though neutrophil accumulation occurred in the livers of APAP-challenged animals, they did not contribute to the initiation and progression of APAP hepatotoxicity (Lawson et al., 2000). One of the studies performed in this thesis was to test if modulation of inflammatory cell recruitment by using aspirin as a pharmacological inhibitor in our APAP, GalN and LPS models of inflammation, could affect the levels of inflammation and toxicity and elicit protection. The aim being that aspirin may be a potential therapeutic tool in protecting against inflammation associated with APAP hepatotoxicity and other drugs associated with DILI. The results of these studies presented in Chapter 3 suggest that aspirin was ineffective in our APAP, LPS and GalN models at reducing the level of toxicity and inflammation and conferred no protection. However, these studies alone provided no evidence for inhibition of the Nalp3 inflammasome, but a collaborative study recently published also looked at the genetic elimination of the Nalp3 inflammasome as well as pharmacological inhibition of Nalp3 with aspirin in APAP-treated mice, found the same ineffective outcome with aspirin, and that elimination of the Nalp3 inflammasome did not protect against APAP hepatotoxicity (Williams et al., 2011). The data presented in Chapter 3 strongly supports recent literature evidence and the conclusion that the activation of the Nalp3 inflammasome and subsequent involvement of neutrophils has no relevant impact on APAP hepatotoxicity and therefore may not be a viable therapeutic target to treat APAP overdose. However, confirmation of these results in the human pathophysiology is required before dismissing this as a therapeutic target in APAP overdose patients. Caution must be exercised in translating this concept to man with respect to aspirin acting as a hepatoprotective agent against APAP-induced liver injury,

6.4 Effect of modulation of HMGB1 on DILI and inflammation

The release of damage-associated molecular pattern (DAMP) molecules from damaged or dying cells following injury has been well described (Bianchi et al., 2007; Martin-Murphy et al., 2010). DAMPs that have been reported to be released in response to APAP hepatotoxicity are heat shock proteins (HSPs), DNA and HMGB1 (Jahr et al., 2001; Scaffidi et al., 2002;

Martin-Murphy et al., 2010). HMGB1 can be passively released by necrotic cells in a hypo-acetylated form in the context of experimental DILI (Antoine et al., 2009), or secreted by activated macrophages in a hyper-acetylated form (Bonaldi et al., 2003). Both forms have been detected in the plasma after an overdose of APAP (Scaffidi et al., 2002). Increased levels of serum HMGB1 have also been demonstrated in patients with APAP- and non-APAP-induced acute liver injury (Craig et al., 2011). Once released, HMGB1 can act as a potent inflammatory mediator which has been shown to link the necrotic cell death of hepatocytes to the inflammatory response (Scaffidi et al., 2002), by targeting TLRs (Yu et al., 2006) and RAGE (Kokkola et al., 2005). It was therefore hypothesised that HMGB1 is an important modulator of inflammation and APAP-induced liver injury and that its inhibition may be a key to improving clinical outcomes.

The studies carried out in Chapter 4 involved using ethyl pyruvate (EP) as another potential pharmacological inhibitor of DILI and inflammation. EP is a potent antioxidant and free-radical scavenger and has been reported to be an effective anti-inflammatory agent in a variety of *in vitro* and *in vivo* model systems (Fink et al., 2007). EP was shown to inhibit LPS-induced NF-κB activation in cultured RAW264.7 murine macrophage-like cells, and reduces HMGB1 release and TNF-α gene expression both in vitro and in vivo (Sims et al., 2001; Ulloa et al., 2002; Yang et al., 2002b). One of the studies presented in this thesis was to see if EP could reduce inflammation in both our LPS and APAP models and if it could subsequently protect against APAP hepatotoxicity by potentially modulating early (TNF-α, IL-6 and IL-1β) and late (HMGB1) cytokine release. The effects of EP on APAP toxicity and inflammation were compared to those seen using an anti-HMGB1 antibody in an APAP model.

Pretreatment with EP significantly reduced the release of HMGB1 in both LPS and APAP models, however, it was not complete inhibition compared to the level of inhibition observed with the anti-HMGB1 antibody. EP pretreatment also significantly reduced LPS and APAP-serum cytokine release. However, despite the reduction in both HMGB1 and cytokine release, pretreatment with EP gave no significant protection in terms of APAP hepatotoxicity. This finding requires further investigation to deduce why the early dose of EP did not protect against APAP hepatotoxicity when EP is known to have antioxidant and free-radical

scavenging properties. EP is a potent antioxidant and free-radical scavenger (Varma et al., 1998; Sims et al., 2001), with antioxidant treatment reported to protect against APAP hepatotoxicity (Oz et al., 2004). EP had no effect on APAP-mediated hepatic GSH depletion in our APAP model. EP is lipophilic, thereby diffusing readily into cells, potentially acting on both intracellular and extracellular radicals. Also, EP is a stable derivative of pyruvate, potentially leading to prolonged effects of this compound after administration. This was confirmed by a study showing pre-treatment of mice with EP provided durable protection against some of the deleterious effects of LPS, even when the duration between the last dose of EP and the endotoxic challenge was 6hr (Sappington et al., 2003). This fact rules out the possibility of EP being rapidly eliminated and subsequently having insufficient levels to affect the release of late-appearing cytokines post LPS or APAP dose. Further studies conducted in Chapter 4 involved a delayed treatment of EP in the LPS and APAP models, to study if any pharmacological effects of EP can persist after waiting for a prolonged period (4hr) after the dose of LPS or APAP. The results showed that delayed treatment of EP conferred a significant reduction in HMGB1 release but not in the release of early cytokines in both LPS and APAP-treated animals. Delayed EP treatment, however, conveyed significant protection to APAP toxicity. A limitation of this work in these models is that the form of HMGB1 released is undetermined. As it has been shown that EP reduces actively released HMGB1 (Ulloa et al., 2002), a future study that incorporates mass spec analysis of HMGB1 in our models is required to show that the cell death released form of HMGB1 (hypoacetylated) is not altered by EP. These findings could be accounted for by the fact that treatment began after the initial toxicity and early peak in cytokines, and that the protection by EP may be due to it targeting the late acting mediator of inflammation, HMGB1 and not by having an antioxidant effect early during APAP bioactivation. It is also worth noting that the protection against APAP hepatotoxicity was greater in anti-HMGB1 treated animals that completely inhibited HMGB1, compared to the partial inhibition of HMGB1 by EP.

EP warrants further evaluation as it may have a therapeutic potential in the clinic for inflammation associated with APAP-induced hepatotoxicity. EP is likely to have a lower propensity to be harmful to humans, with its close similarity to the endogenous metabolite pyruvate, its safety profile in animals and its common use as a food supplement in humans. EP has been tested in human volunteers and shown to be safe at clinically relevant doses (Fink, 2008). Most importantly, from a clinical standpoint EP appears to have beneficial

effects when it is administered after toxicity has become well established after APAP overdose. Overall, EP may be a useful adjuvant to other treatments for DILI, by targeting the inflammatory signalling pathway and thereby potentially protecting against the inflammatory-mediated exacerbation of liver injury.

6.5 Expression of RAGE in APAP hepatotoxicity

The observations in Chapter 4 and in the literature suggest that HMGB1 signalling may be an important factor in the progression of APAP-induced liver injury by activation of the immune response (Scaffidi et al., 2002). Chapter 4 focused on the release of HMGB1 and the therapeutic potential of blocking this signalling pathway. Downstream from this, experimental studies have given evidence that the interaction of HMGB1 with its receptor RAGE, contributes to the pathophysiology of inflammatory conditions. RAGE exists as a full-length isoform (flRAGE) that is bound to the membrane and as a soluble form derived from alternate splicing (esRAGE). RAGE is commonly associated and often cited as a molecule that can mediate and exacerbate inflammatory conditions such as diabetes, atherosclerosis and neurodegenerative diseases as ligand engagement of RAGE leads to prolonged inflammation by induction of NF-κB, resulting in RAGE-dependent expression of proinflammatory mediators. It is well known that RAGE expression is elevated in response to the increased level of its ligands and has the potential to perpetuate inflammation by a positive feedback effect on RAGE expression (Schmidt et al., 2000).

One of the aims of this thesis was to evaluate the expression of RAGE in the tissues of APAP-treated mice and its potential relationship to the pathophysiology of APAP hepatotoxicity. The results of a 24hr APAP time course study in Chapter 5 showed that in fed mice, RAGE mRNA of total, full-length and endogenous secretory forms of RAGE increased between 1hr and 3hr, with basal levels of each form observed at preceding and later time points. A change in expression of RAGE mRNA was observed in fasted mice, with a more prolonged increase in expression of the three isoforms culminating in a peak of expression at 5hr. A possible explanation for these findings is linked to the effect of fasting on inflammation observed in Chapter 2, in that fasted animals had a greater inflammatory response during APAP hepatotoxicity. These results suggest that the level of expression of

RAGE mRNA increases in more pronounced toxic and inflammatory conditions. The pathological role of RAGE in APAP hepatotoxicity may be mediated through a complex interplay between the RAGE isoforms. The presence of more flRAGE presumably results in greater and more sustained signalling through the flRAGE receptor, whereby esRAGE may be upregulated in response to this to act as a negative feedback mechanism as esRAGE can act as a decoy for RAGE ligands and thereby inhibit the signalling pathway, suppress overexpression of flRAGE, and perpetuate inflammation. In contrast to the results of the expression of mRNA, RAGE showed a different pattern of expression with respect to protein. There was no difference in expression of RAGE protein between control and APAP-treated animals at any of the time points post APAP-dose. As the flRAGE form has been shown to be proteolytically cleaved to generate a soluble form (Raucci et al., 2008) and that the membrane-bound RAGE can be internalised (Sevillano et al., 2009), it could be that changes in RAGE expression at the protein level are not detected. As the dynamics of RAGE mRNA and protein turnover are not yet fully established, further investigation is needed, however, it is worth noting that analysis at the mRNA level does not always correlate well with changes in protein levels (Greenbaum et al., 2003).

The full extent of RAGE activation and expression in response to APAP hepatotoxicity requires further investigation in order to accurately evaluate the contribution of RAGE in the overall pathogenesis of APAP hepatotoxicity. The down-regulation of RAGE expression or blockade of RAGE downstream signalling may be a promising target for therapeutic intervention. There is evidence supporting a role of RAGE in the pathogenesis of APAP hepatotoxicity as it was shown that its inhibition by sRAGE resulted in increased survival and a reduction in oxidative stress in mice (Ekong et al., 2006). Further evaluation of the therapeutic potential of RAGE is required, however, the potential for inhibiting RAGE-mediated inflammation in human cases of DILI exists.

6.6 Perspectives on research and future investigations

The rationale of the work undertaken in this thesis was to provide further evidence for the role of inflammation during DILI using a variety of *in vivo* models. Support for the hypothesis that immune cells can play a critical role in drug-induced hepatotoxicity is

steadily growing. There is increasing evidence to support that in many cases, the effects of drugs on cells within the liver can act as an initiating event for an immune response that can determine the overall extent of liver injury. Therefore, it is important for a greater understanding of the mechanisms by which cells of the innate immune system are activated and recruited into the liver during DILI and how they exacerbate the overall pathogenesis. The work presented in this thesis was aimed at using the current knowledge collected from the literature to develop a set of *in vivo* models that could be used to assess both basic and novel mechanisms of inflammation, with the aim of testing potential therapeutic strategies. Table 6.2 summarises the various models, therapies and mechanisms that were developed and/or assessed within this thesis.

Table 6.2: Overview of inflammatory models, therapies and mechanisms assessed within this thesis.

In vivo models of inflammation	**Potential therapies (Inhibitors)**	**Novel mechanisms**
- APAP - GalN - LPS - Fasting	- Aspirin - Anti-HMGB1 antibody - EP	- RAGE expression - RAGE in nucleus
Aims - Test mechanisms of inflammation - Test potential therapies - Assess new mechanisms - Translate to human risk assessment?	**Aims** - Test/probe mechanisms - Assess inflammatory suppressors	**Aims** - Assess new mechanism

Our current knowledge of the mechanisms of DILI is that initial hepatocyte damage leads to downstream events such as the activation and recruitment of immune cells, release of inflammatory cytokines and chemokines and also initiation of an adaptive immune response. An effective way towards a greater understanding of these molecular and cellular mechanistic events is by developing animal models of DILI that incorporate many of the inflammatory

pathways. The murine model of APAP hepatotoxicity has been widely studied to gain insight into the mechanisms of DILI as it has similar characteristics to human patients based on clinical, biochemical and histological observations (Hinson et al., 1981; Ishida et al., 2002). It has been shown that the concordance of hepatic toxicities between animals and humans is ~50% (Olson et al., 2000) therefore, the development of models that more accurately represent the human physiological situation is required. APAP was used as a primary model of DILI in this thesis to evaluate the inflammatory response with the prospect that any observations could be translated to man. GalN and LPS were also used as models as both could induce a specific response. GalN was characterised as inducing part hepatotoxicity, part inflammation, whereas the LPS model had no hepatotoxic component but was mainly systemic inflammation. These models, when compared to APAP gave a wider view on the mechanisms of inflammation, which allowed for further exploration with regards to potential inhibitory mechanisms in later studies. APAP and GalN, served as useful models that could represent both a toxicological and inflammatory response in man, while the LPS model served as a positive control for inflammation, in which a potent systemic inflammatory response could be observed. The limitation of the LPS model is that it does not represent DILI in man, as it is a model of sepsis and endotoxaemia, but has been extensively used in rodent studies as a model to investigate innate immune mechanisms (Salojin et al., 2006; Roger et al., 2009; Wang et al., 2010). The models used in this thesis have given a broad spectrum for the evaluation of proinflammatory activity, each having a different mechanism of action for producing inflammation.

Some of the mechanisms explored in this thesis using these models were based on immune cell recruitment and signalling. Based on accumulating evidence in the literature, immune cells can play an important role in the progression of most but not all cases of DILI. While it is widely regarded that neutrophils play a detrimental role in conditions such as ischaemia-reperfusion or cholestasis (Gujral et al., 2003b; Jaeschke et al., 2003b; Hasegawa et al., 2005), there has been controversy regarding their role in APAP hepatotoxicity (Bauer et al., 2000; Lawson et al., 2000; Cover et al., 2006). While mouse models have been extensively used to dissect the mechanism of inflammation, it must be taken into consideration that there are differences between the innate immune system of mice and humans (Mestas et al., 2004; Fairbairn et al., 2011). An example of the differences can be highlighted by the cellular constituents of the blood, where 50–70% of the population of peripheral blood cells in

humans is neutrophils but only 10–25% in the mouse (Doeing et al., 2003; Mestas et al., 2004). It is therefore important to consider that the results and conclusions of the mechanisms of inflammation drawn from these mouse models need to be investigated further and translated in DILI patients before therapeutic interventions can be successfully determined. Preclinical *in vivo* models that incorporate inflammatory cells are critical to address all aspects of DILI. The work undertaken in this thesis aimed to address this by modulating immune cell recruitment and signalling. The evaluation of proinflammatory cytokine production is useful in assessing an inflammatory response as they can be easily measured in the serum. The limitation to evaluating these cytokines in the serum is that their release is not restricted to specific cells. Serum biomarkers that monitor the function of specific cells such as neutrophils and macrophages need to be identified and established in models of DILI. DAMPs such as HMGB1 have potential as a biomarker of inflammation as the acetylated form is released from activated immune cells (Bonaldi et al., 2003). An improvement to the models used in this thesis would be to incorporate the measurement of HMGB1 alongside the existing cytokine and histopathological analysis. Evaluation of liver histopathology along with standard clinical pathology markers of liver injury currently represents the most reliable method for the assessment of hepatotoxicity in standard toxicology studies (Olson et al., 2000). However, inconsistencies when comparing ALTs to histological findings were evident in some of the studies presented in this thesis. The need for biomarkers that better represent histological findings is evident. HMGB1 is a non-organ specific biomarker of necrosis and inflammation and is a potential non-invasive biomarker that correlates to histopathology (Antoine et al., 2009). Future investigations should incorporate the measurement of HMGB1 alongside clinical chemistry parameters and histological assessment.

The need for improved animal models of DILI that more closely resemble the human physiological situation is evident. An increased understanding of the biological mechanisms of inflammation associated with DILI and their relevance to humans should lead to increased use and application of data from animal models to human health risk assessments. Developing models that use perturbed animals may more accurately predict and determine human risk. For example, the use of fasted animals in this thesis may represent a more clinically relevant model with respect to DILI and inflammation in human patients. The nutritional state of patients was shown to be an important factor in APAP hepatotoxicity (Whitcomb et al., 1994), where fasting enhanced the prospects of liver injury associated with

therapeutic doses of APAP. Indeed, the results presented in Chapter 2 support this finding as APAP hepatotoxicity was greater in fasted animals compared to non-fasted. Fasting can decrease intracellular GSH, increasing the overall susceptibility to APAP toxicity (Pessayre et al., 1979). This situation can resemble human conditions in which malnutrition is associated such as chronic alcoholism and anorexia. An alcoholic or individual suffering from anorexia nervosa may already have reduced GSH levels in the liver and may therefore be at increased risk of developing toxic liver injury with APAP (Riordan et al., 2002; Zenger et al., 2004). In these particular cases, the impact of fasted animal models on the mechanism of APAP-induced liver injury and the resulting inflammatory response may closely resemble the human situation and could have important implications in translating the results to the clinic and in the risk assessment for humans. Another perturbed animal model to take into consideration utilises LPS co-administration with hepatotoxic agents to induce an inflammatory background. In these models, LPS exposure induces a mild inflammatory response that can synergistically augment hepatotoxicity in the presence of hepatotoxic drugs, usually at non-injurious doses. Examples where this has been demonstrated include LPS-co-administration with APAP (Maddox et al., 2010) and GalN (Lawson et al., 1998). The mechanisms of these models are similar in which LPS-generated inflammation can lead to recruitment and activation of neutrophils in the liver, triggering exacerbation of injury. The relevance of these models to man is that the presence of inflammation may influence the pathogenic outcome of chemical exposure. Patients suffering from pre-existing inflammatory conditions or bacterial or viral infections may be at higher risk of developing hepatotoxicity at therapeutic doses of particular drugs. This model can mimic the inflammatory episodes that can occur in man and has the potential to aid human risk assessment although further investigation is required. LPS-co-administration would be a useful additional model to the work carried out in this thesis and should be considered for future investigations into mechanistic studies of DILI. The application of these perturbed animal models to human risk assessment will require interpretation of the data with respect to its relevance to humans, as well as recognition of the limitations of these models but overall could provide a more physiologically relevant model to human DILI.

To further understand the activation of the innate immune system in response to DILI, the interaction between extracellular signals such as DAMPs and receptors must be addressed. Using the variety of models developed and characterised in this thesis, further exploration of

these signalling pathways was conducted by testing potential therapies that target a specific part of the inflammatory pathway. Various agents were used to modulate either immune cell recruitment or their signalling, with the aim to determine if they could provide protection against DILI. Aspirin and EP were selected as pharmacological inhibitors due to reports in the literature of their effects at modulating inflammation (Ulloa et al., 2002; Imaeda et al., 2009). Also, as both are safe for human consumption, there was the potential to translate any beneficial effect to the clinic. Overall it was determined that aspirin had no significant effect on inflammation in our models, with no notable protection to DILI. These results were supported by a recent study by Williams et al. (2011). A potential future investigation to consider may involve changing the route of administration of aspirin, as well as delaying treatment until after hepatotoxicity is evident, as pretreatment in the drinking water for 3 days before drug treatment at a range of doses had no effect. Delaying treatment of aspirin may be more clinically relevant as pretreatment would not be feasible for intentional or non-intentional overdose patients. EP had a more promising outcome, as both pre- and delayed treatment resulted in a significant reduction in HMGB1 release. Mass-spec analysis is required to confirm that the HMGB1 being inhibited in our models is the acetylated form which is actively released by immune cells. Delayed treatment of EP appeared to reduce the level of APAP hepatotoxicity as determined by ALT activity, suggesting that EP could be a potential therapeutic treatment in APAP hepatotoxicity. It should be noted that EP treatment, compared to the effects of the more specific anti-HMGB1 antibody was less effective in reducing toxicity. Further studies should be targeted towards testing a range of hepatotoxic agents with EP to see if there is a similar outcome. A limitation to the modulatory studies in this thesis was that, anti-HMGB1 antibody aside, the pharmacological treatment strategies of aspirin and EP were not specific to the targets for which they were intended. While the off-target effects of these agents need to be investigated, the lack of specificity could result in poor efficacy if translated to the clinic.

One of the aims of the thesis was to investigate the novel mechanism of RAGE expression during APAP hepatotoxicity. In summary, RAGE interaction with its ligands leads to an inflammatory response resulting in RAGE-dependent expression of proinflammatory mediators and upregulation of RAGE by a positive feedback loop (Schmidt et al., 2000; Bierhaus et al., 2001). The studies performed in Chapter 5 aimed to address the hypothesis that RAGE expression was modulated in response to APAP toxicity. This work served to

gain further mechanistic understanding of inflammation associated with DILI, using our APAP model. A novel finding in these studies was that RAGE was found to be localised in the nucleus of a small number of hepatocytes post APAP treatment. This finding requires further investigation to determine if this is a functional response by cells to sequester RAGE in the nucleus to prevent further inflammatory signalling, thereby acting as a negative feedback loop to prevent prolonged inflammation. Functional blockade of RAGE by application of recombinant RAGE significantly diminished liver damage according to a study by Ekong et al. (2006). Future studies should involve utilising recombinant RAGE and other low molecular weight inhibitors of RAGE such as telmisartan (Nakamura et al., 2005) in our models to attempt to elucidate the effects of blocking this receptor on inflammation associated with DILI and the overall pathophysiology. Inhibition of the HMGB1/RAGE interaction may provide a new possibility for the treatment of DILI and requires further investigation. Whether RAGE inhibitors will be useful at the later stages of APAP-induced liver injury or in other cases of DILI remains to be determined.

6.7 Concluding remarks

In summary, the experiments planned within this thesis were designed to further address our understanding of the role of the innate immune system and inflammatory response during DILI. In particular, the aims of the investigations presented in this thesis were to assess the contribution of inflammation by targeting certain points in the signalling pathway between necrotic hepatocytes and cells of the innate immune system. However, due to the continually increasing complexity of the innate immune and inflammatory-mediated liver injury mechanisms as well as some of the controversies in the literature, not all aspects of this field could be covered in depth. As DILI is a significant burden for drug development in addition to morbidity and mortality, a preclinical *in vivo* model to incorporate inflammatory cells to assess immune responses is essential to address all potential aspects of DILI. One of the aims of this thesis was to have a set of *in vivo* models that could be utilised to investigate simultaneously the molecular mechanisms involved in hepatotoxicity and inflammation. The use of model hepatotoxins APAP and GalN and the inflammation-inducing endotoxin LPS, as tools to induce a specific response, have proved capable of assessing the pathway associated with DILI and inflammation, and the potential of pharmacological agents to

modulate inflammation by targeting specific parts of the signalling pathway. One novel finding in this thesis was the potential for EP to be used as a pharmacological inhibitor of DILI-associated inflammation in mice; however, further work is required to translate this to the clinic. Another was the expression of RAGE in the nucleus of hepatocytes during DILI. The full implications of the mechanism of the inflammatory response during DILI and the subsequent consequences in the liver still remain to be elucidated, but the work presented in this thesis has provided evidence to suggest that the overall contribution of the innate immune and inflammatory responses during APAP induced hepatotoxicity is minimal.

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